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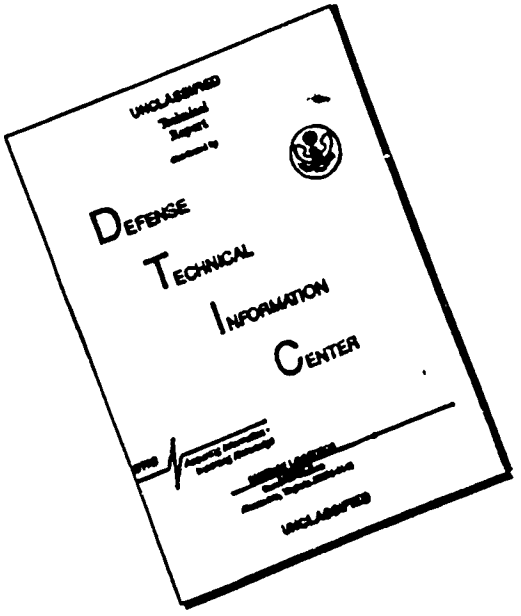


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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The goal of this work is to develop novel, efficacious, injectable, gene-specific therapeutics for treatment of human immunodeficiency virus (HIV) infection. These products will be nuclease resistant, stereospecific antisense inhibitors of human immunodeficiency virus gene expression and activity. Antisense DNA inhibition has worked in this laboratory against several human and viral genes in cell culture.

In this project, routes are being developed for the stereospecific synthesis and purification of all-S or all-R oligodeoxynucleoside methylphosphonates directed against specific HIV mRNAs, and against *tat* protein. Several dimers have been prepared by the pentavalent solution route, and encouraging results have been obtained for the trivalent solution route.

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The resulting oligomers have been characterized by fast atom bombardment mass spectroscopy in the laboratory of Dr. Julie Leary (Berkeley), and high field nuclear magnetic resonance spectroscopy, at USF and in the laboratory of Dr. David Gorenstein (Purdue). Oligomer toxicology and pharmacology have been studied in mice, in collaboration with Dr. Ralph Brinster (Pennsylvania). Doses of 15 and 50 mg/kg of pentadecadeoxynucleoside methylphosphonates were not toxic, and displayed multiphasic pharmacokinetics in plasma.

Oligomers targeted against *tat* were tested for their efficacy in preventing expression of a *tat*-dependent reporter gene in the laboratory of Dr. Lee Bacheler (DuPont), and preventing expression of HIV p24 and syncytial formation in HIV-challenged cells in the laboratory of Dr. Flossie Wong-Staal (NCI).

The mode of interaction between HIV-1 transactivating *tat* protein and the trans-acting responsive (TAR) sequence in HIV mRNAs, which results in *tat* stimulation of HIV mRNA transcription and translation, was studied *in vitro* with synthetic *tat* protein. The synthetic *tat* peptide was crossreactive with anti-*tat* antiserum, in the laboratory of Dr. David Baltimore (MIT), but was nonspecific in its binding to a series of RNA transcripts.

## D. SUMMARY

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## E. FOREWORD

Acquired immune deficiency syndrome is the result of infection by a retrovirus called human immunodeficiency virus (HIV). The genome of HIV extends about 9200 bp, including long terminal repeats (LTRs) at each end (Ratner, et al., 1985; Wain-Hobson, et al., 1985; Sanchez-Pescador, et al., 1985). The genome includes the common retroviral genes for group specific antigens, *gag*, polymerase enzymes, *pol*, and envelope proteins, *env*. In addition, there are two less well characterized large open reading frames: *vif*, which is just 3' of the *pol* transcription unit, and appears to be required for viral infectiousness and replication (Fisher, et al., 1987); *nef*, which extends from the 3' end of *env* into the LTR, acts like a negative regulatory factor and resembles the *ras* oncogene p21 protein (Guy, et al., 1987).

A bipartite sixth gene, *tat*, occurs in the *env* region, but in a different reading frame (Sodroski, et al., 1985; Arya, et al., 1985). *tat* encodes a doubly spliced mRNA for an 86 residue protein which shows homology to nucleic acid binding zinc-finger domains in other proteins (Berg, 1986), particularly Cys-Xaa-Xaa-Cys, which occurs four times. The 14 kD *tat* protein increases the level of transcription and the efficiency of translation of HIV mRNA, and hence HIV replication (Rosen, et al., 1986; Fisher, et al., 1986; Cullen, 1986; Wright, et al., 1986). Hence, inhibition of *tat* expression may be especially effective at preventing production of infectious virions in IIIV-infected cells.

The *tat* protein interacts directly or indirectly in *trans* with the first 80 nt of all HIV mRNAs, the transactivation responsive (TAR) element (Rosen, et al., 1985). This sequence was calculated to form a tight hairpin loop from the cap to nt 59, so perhaps the *tat* protein is an RNA helix destabilization protein which opens up the 5' end of HIV mRNAs, thus increasing their translational efficiency (Okamoto and Wong-Staal, 1986); this model is analogous to an earlier model for the mode of action of prokaryotic translational initiation factor 3 (Wickstrom, 1974). The predicted secondary structure, and a second stable hairpin loop in the TAR were confirmed by nuclease probing; the sequence and structure are necessary for *tat* activity (Muesing, et al., 1987). Immunofluorescence analysis implied that *tat* protein is primarily located in the nucleus, suggesting that any protein-RNA interactions would probably occur in the nucleus, by stimulating transcription, anti-termination, processing, or transport to the cytoplasm (Hauber, et al., 1987; Kao, et al., 1987).

Synthetic oligodeoxynucleotides have been successfully applied by several laboratories to inhibit gene expression (rev. by van der Krol, et al., 1988). Encouraging results have been obtained in this laboratory for VSV matrix protein (Wickstrom, et al., 1986), and human c-*myc* oncogene (Heikkila, et al., 1987; Wickstrom, et al., 1988, 1989; Bacon, et al., 1989; Bacon and Wickstrom, 1989). In the case of HIV, antisense oligodeoxynucleotides directed against sites in the LTR or against splice junction sites were effective in the 20 $\mu$M range for inhibiting HIV reverse transcriptase activity and p15 and p24 expression in H9 cells challenged by HIV in culture (Zamecnik, et al., 1986). The most recent HIV work from Zamecnik's laboratory confirms their earlier observations, and makes clear that antisense oligodeoxynucleotides are efficacious against a variety of targets, at doses which are nontoxic in mice (Goodchild, et al., 1988). In the work presented below, this laboratory has also observed some efficacy of antisense oligodeoxynucleotides against HIV *tat*.

Oligodeoxynucleotides themselves are not stable enough for intravenous or oral administration. Uncharged methylphosphonate oligodeoxynucleosides are resistant to nucleases, enter animal cells more efficiently than charged oligodeoxynucleotides, and specifically inhibit expression of simian virus 40 (Miller, et al., 1985) and VSV (Agris, et al., 1986), rabbit globin (Blake, et al., 1985), herpes simplex virus 1 (Smith, et al., 1986), and HIV (Sarin, et al., 1988). However, relatively high concentrations are required for significant inhibition. One would expect that the greater longevity, improved cellular uptake, and lack of charge on oligodeoxynucleoside methylphosphonates would make them much more effective inhibitors of mRNA translation than normal oligodeoxynucleotides. However, in the case of dihydrofolate reductase mRNA translated in the same system, oligodeoxynucleoside methylphosphonates were over 100 times less efficient than normal oligodeoxynucleotides (Maher and Dolnick, 1988).

The poor hybridization of oligodeoxynucleoside methylphosphonates is probably due to the existence of R and S diastereomers at each phosphodiester bond (Kan, et al., 1980). For example, in a normal octamer with a single methylphosphonate bond, it was found that the oligomer with an R bond had a higher melting temperature than the oligomer with an S bond (Bower, et al., 1987). For the related ethyl phosphotriesters, Abramova, et al. (1988) observed that alkylation of poly(dA), and of poly(rA) tails in Krebs ascites cells, by an aryl nitrogen mustard derivative of tetrathymidyluridine was about 20 times as efficient for one particular diastereomer at each bond versus the other. The point is that a large difference in efficacy was apparent even for a tetramer, as we expect for oligodeoxynucleoside methylphosphonates. They have subsequently determined that R diastereomers of methylphosphonates, alternating with normal diesters, impart greater efficacy (Amirkhanov and Zarytova, 1988). In the work presented below, this laboratory has made some progress in stereospecific synthesis of oligodeoxynucleoside methylphosphonates. It has also been found that racemic oligodeoxynucleoside methylphosphonates may be injected into mice at 50 mg/kg without obvious toxicity, and recovered intact from the blood for at least four hr. after administration. An oligomer specific for c-*myc* strongly down-regulated c-*myc* p65 expression in circulating lymphocytes of c- *nyc* transgenic mice.

The synthesis of oligomers of $\alpha$-deoxynucleotides, instead of the normal $\beta$-deoxynucleotides, also has potential for achieving nuclease resistance without loss of base pairing effectiveness (Morvan, et al., 1986; Bacon, et al., 1988). However, while the unusual $\alpha$-oligodeoxynucleotides hybridize in parallel with mRNA even more tightly than normal $\beta$-oligodeoxynucleotides hybridize in antiparallel, they show little efficacy for hybrid arrest (Gagnor, et al., 1987). In the work presented below, this laboratory has observed that $\alpha$-oligodeoxynucleotides survive very well in mammalian sera, with half-lives on the order of 12 hr. (Bacon, et al., 1988).

Study of RNA structure and RNA-protein interactions has been a longterm interest of this laboratory (Wickstrom and Laing, 1988). Hence, the potential for RNA binding by *tat* protein stimulated great interest. Frankel, et al. (1988) have isolated HIV-1 *tat* protein which was overexpressed in bacteria, and analyzed its metal binding and oligomerization. They observed 2 $Cd^{2+}$ or $Zn^{2+}$ ions per *tat* monomer, but weak $Co^{2+}$ binding, and proposed a dimeric structure for active *tat*. In their hands, the recombinant protein bound both DNA and RNA nonspecifically. This result is paradoxical, since *tat* was first characterized by its dependence on the TAR region for activity (Rosen, et al., 1985), and sequence mutants in the TAR stem-loop structure abolish *tat* activity (Feng and Holland, 1988). In the work presented below, this

laboratory has found similar results in gel mobility shift experiments at low ionic strength with a synthetic *tat* polypeptide, and no specific footprinting onto an HIV first exon transcript at physiological ionic strength (Zou, et al., 1989).

It is anticipated that antisense oligomers will display some cytotoxicity, making them inappropriate for chronic use in an animal or human system. Furthermore, prolonged use may well select for clones which overexpress the mRNA target of the antisense oligomer; this problem may not arise if days or weeks of exposure is sufficient to commit transformed cells to differentiate. The possibility of short term treatment will be made more plausible if antisense therapy against active viral genes also induces re-expression of major histocompatibility complex class I antigens. Interestingly, the related Ad12 E1A and BK large T antigens have been implicated in down regulation of MHC class I antigen expression in transformed human cells (Vasavada, et al., 1986). Completing the analogy, down regulation of MHC class I antigens has also been observed in human Burkitt's lymphoma cells transformed by c-*myc* translocated to an immunoglobulin locus (Masucci, et al., 1987). The importance of these findings lies in the fact that cells which express little or no MHC class I antigens are resistant to cytolytic T lymphocytes specific for antigens expressed by those cells, resulting in a loss of immune surveillance. Similarly, MHC I expression in human melanoma cell lines was observed to be inversely correlated with c-*myc* expression (Versteeg, et al., 1988). Hence, it is possible that reducing the level of viral mRNA translation may not only halt viral replication and the spread of infection, but even re-establish immune surveillance of virally transformed cell populations. In preliminary work presented below, this laboratory has found significant differences between MHC class I levels in normal and transformed cells.

The most frequent question asked about antisense oligodeoxynucleotide hybrid arrest experiments focuses on how the oligodeoxynucleotides enter the cells. Anecdotal accounts of inhibition of uptake by dinitrophenol or cytochalasin B imply an ATP-mediated mechanism, i. e., active uptake (J. Goodchild, at EMBO/INSERM Antisense Regulation Workshop). Vlassov, et al. (1986) have observed uptake kinetics which may be construed to imply uptake by simultaneous adsorptive endocytosis, stimulated endocytosis, and pinocytosis. Bennett, et al. (1988) have been characterizing a human leukocyte cell surface protein, about 30 kDa, which binds large DNA molecules and mediates their internalization and degradation to oligodeoxynucleotides. This protein is a candidate for a DNA receptor, and Dr. Bennett has provided us with samples of his monoclonal antibodies 12A and 24T in order to determine whether or not the putative receptor takes part in oligodeoxynucleotide uptake. In preliminary work presented below, this laboratory has found evidence for the presence of these receptors on human hematopoietic cells.

## F. TABLE OF CONTENTS

A. Front Cover . . . . . 1

B. Report Documentation Page . . . . . 2

D. Summary . . . . . 3

E. Foreword . . . . . 4

F. Table of Contents . . . . . 7

G. Body of Report . . . . . 8

H. Literature Cited . . . . . 50

I. Publications . . . . . 53

J. Personnel . . . . . 56

## G. BODY OF REPORT

1. *Antisense Oligodeoxynucleotide Inhibition of c-myc Oncogene Expression* (Reprints and Preprints in Appendix)

A calculated secondary structure for c-*myc* mRNA placed the initiation codon in a bulge of a weakly basepaired region, accessible for antisense arrest. Treatment of PHA-stimulated normal human peripheral blood lymphocytes with an antisense oligomer against the predicted bulge resulted in sequence-specific, dose-dependent inhibition of p65 expression, mitotic index, and entry into S phase (Heikkila, et al., 1987).

Furthermore, treatment of HL-60 cells with the anti-c-*myc* oligomer yielded sequence-specific, dose-dependent inhibition of both p65 expression and proliferation (Wickstrom, et al., 1988), and induced differentiation along the granulocytic pathway (Wickstrom, et al., 1989). Efficacy was three times greater than with normal cells. Oligomer uptake by HL60 cells levelled out at about 1-2% of the labelled oligomers, which survived intact for up to 24 hr. In contrast, oligomers remaining in the culture medium supernatant disappeared by 8 hr. Indirect immunofluorescence and radioimmunoprecipitation assays of HL-60 cells treated with anti-c-*myc* oligomer revealed sequence specific, dose dependent inhibition of p65 expression.

Daily addition of anti-c-*myc* oligomer to HL-60 cells growing in a serum-free medium is as effective as 1% $Me_2SO$ (Bacon, et al., 1989). Mitotic index and colony formation in methocel were similarly inhibited. It thus appears that p65 facilitates replication, which may be halted by antisense oligodeoxynucleotides, directed against a predicted hairpin loop containing the initiation codon of human c-*myc* mRNA.

2. *Stability of α-Oligodeoxynucleotides* (Reprint in Appendix)

We have also collaborated with Dr. Jean-Louis Imbach to study the nuclease resistance of α-oligodeoxynucleotides, which survive with no apparent degradation over 24 hr. at 37° in HeLa cell postmitochondrial cytoplasmic extract or RPMI 1640 medium with 10% fetal bovine serum. Under the same conditions, α-oligodeoxynucleotides are slowly degraded in rabbit reticulocyte lysate, undiluted fetal bovine serum, and undiluted human adult serum with a half-life of roughly 24 hr. (Bacon, et al., 1988).

3. *Synthesis of HIV tat protein* (Draft in Appendix)

The transactivating *tat* protein of human immunodeficiency virus (HIV) displays some characteristics and properties of an RNA binding protein. Analysis of the structure and function of this 86 amino acid polypeptide, and its interactions with RNA, require significant amounts of pure, native protein. The small size of *tat* made its chemical synthesis appear promising. Many problems were posed, however, by automated synthesis of the *tat* sequence. These include a complex Arg and Gln rich segment, and the presence of 7 Cys residues, four of which are thought to be capable of $Zn^{2+}$ binding. These challenges have been solved in an fluorenylmethoxycarbonyl (Fmoc)-mediated synthesis using trimethoxybenzyl side chain protection for Gln, the highly efficient benzotriazolyloxy tris(dimethylamino) phosphonium hexafluorophosphate (BOP) and hydroxybenzotriazole (HOBt) coupling method, use of trityl (Trt) and acetamidomethyl (Acm) moieties for selective Cys protection, advanced expert system predictive software to preassign coupling times, an efficient linker substituted

polystyrene support, and optimized cleavage and deprotection conditions. Peptides removed during the assembly of *tat* at the 20, 36, 53, 73, and final 86 residue steps have been purified by reversed phase liquid chromatography and sequenced. The analytical results confirm the efficiency and fidelity of the synthesis. Immunological crossreaction of the full length peptide was observed with anti-*tat* antiserum.

*4. Oligodeoxynucleoside methylphosphonate synthesis*

Preliminary syntheses of dimethoxytrityl thymidine methylphosphonyl imidazolide and dimethoxytrityl thymidine methylphosphonyl thymidine according to Miller, et al. (1986) were carried out by the PI as a guest researcher in the laboratories of Drs. Jack Cohen and Sam Broder at the National Cancer Institute. Thin layer and liquid chromatography indicated that the syntheses were successful, that the imidazolide is stable in solution for several days, and upon chromatography on $C_{18}$ silica in anhydrous acetonitrile (Fig. 1). NMR spectra confirmed synthesis of the imidazolide (Fig. 13), which successfully coupled to thymidinyl silica, quantitated by detritylation of the dimer silica product. The dimer product was hydrolyzed from the support, and partially resolved on an old $C_{18}$ column (Fig. 2). The evaporated R and S pool gave a correct FABMS pattern (Fig. 9), and was successfully resolved by liquid chromatography on a fresh $C_{18}$ column (Fig. 3). As expected, the fast eluting S peak displayed the larger CD peak, and the slower eluting R peak gave a smaller peak, plus a blue shifted trough (Fig. 4). Hence, reference dimers are available for 2D NMR analysis and comparison with products from new routes. Similarly, the diastereomers of 3',5'-bis(inosine) methylphosphonate have been prepared and separated on a scale of 100 $\mu$mol (Fig. 5).

The first step in preparing a stereospecific reference tetramer is to prepare stereospecific dimers. 5'-O-dimethoxytrityl thymidine methylphosphonyl thymidine-3'-O-acetate was prepared in a one-pot reaction by activating the 3'-OH of 5'-O-DMT-thymidine with methylphosphonyl bis(imidazolide) to the 3'-O-methylphosphonyl imidazolide. Addition of 3'-O-acetyl thymidine yielded the dimer, which was eluted as a racemic mixture from $C_{18}$ silica with 60% acetonitrile/40% water. $^{31}P$ nmr gave peaks at 31.6 and 32.1 ppm from 85% $H_3PO_4$, in agreement with Dorman, et al. (1984). The diastereomers were separated isocratically on silica eluted with 2% MeOH/98% $CHCl_3$ (Fig. 6). The peak eluting at 18.4 min. displayed a $^{31}P$ nmr peak at 31.6 ppm, while the peak eluting at 26.1 min. showed a $^{31}P$ peak of 32.1 ppm (Fig. 16).

To synthesize a tetramer, half of each dimer diastereomer will be deprotected at the 5'-OH, and half at the 3'-OH. The two derivatives will be coupled as for the dimer, yielding a tetramer with stereospecific first and third methylphosphonates, and a racemic second methylphosphonate. The tetramer diastereomers will be separated by liquid chromatography as before. We have now prepared tetrathymidine methylphosphonates from the stereospecific dimer of peak I in Fig. 5. This preparation separated into two closely spaced peaks upon liquid chromatography (Fig. 7). We are awaiting FABMS and NMR results to see whether the peaks represent the all-S and S-R-S tetramers, respectively.

For the trivalent route, 5'-dimethoxytrityl thymidine methylphosphinyl diisopropylamide has been prepared according to Dorman, et al. (1984), and its coupling rate to 3'-O-benzoyl thymidine was found to be quite rapid by $^{31}P$ NMR (Fig. 17). The expected $^{31}P$ spectra were also obtained for 5'-dimethoxytrityl thymidine methylphosphonyl diisopropylamidite reacting with 5'-activated 3'-O-acetyl thymidine in

the **absence** of tetrazole (Fig. 18). Tetrazole was eliminated expressly to prevent epimerization, and its catalytic role was substituted by a proprietary 5' activation of 3'-O-acetyl thymidine. The diastereomers of the activated trivalent monomer may be separated on $C_{18}$ silica (Fig. 8), but would best be separated anhydrously on silica (or a less reactive matrix) before carrying out the same 5'-activated coupling. Only then will it be known if this route will allow stereospecific trivalent coupling.

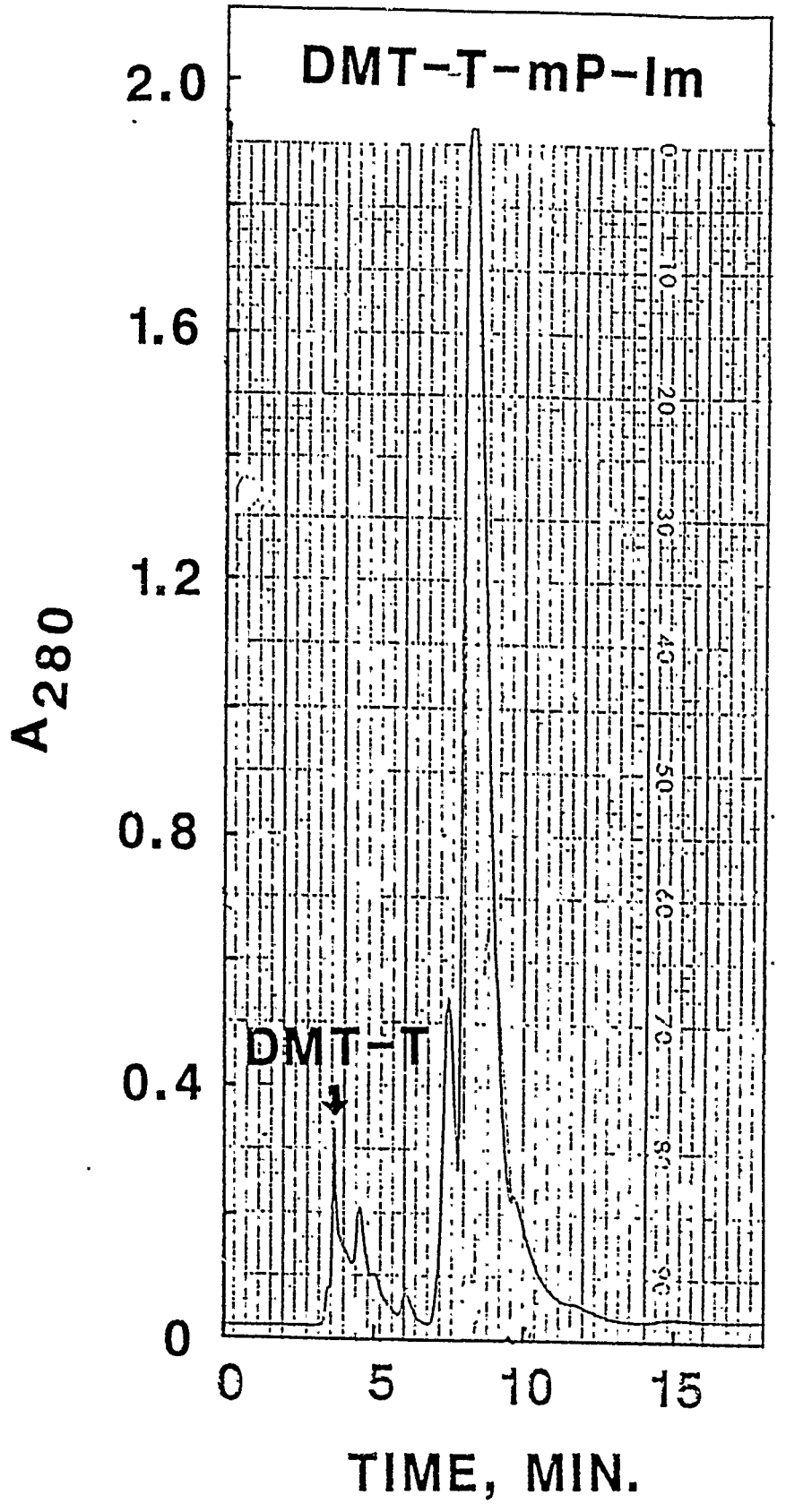


Fig. 1. Liquid chromatography of DMT-T methylphosphonyl imidazolide on a 4.6 x 250 mm column of $C_{18}$ silica in anhydrous acetonitrile at a flow rate of 1.0 mL/min.

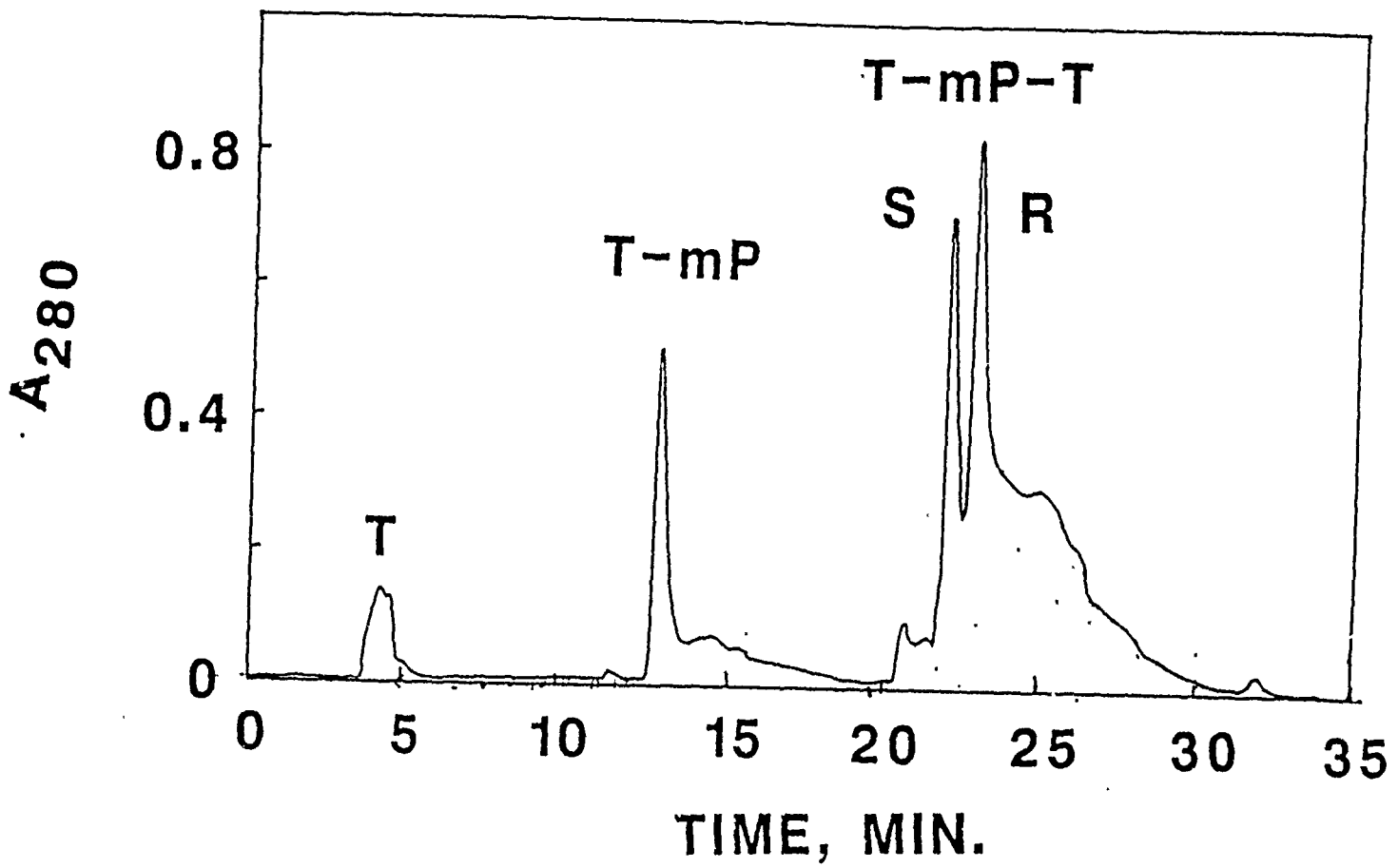


Fig. 2. Liquid chromatography of crude T-T methylphosphonate on a 4.6 x 250 mm column of $C_{18}$ silica, eluted with a gradient of 0-50% acetonitrile in water, 1%/min., at a flow rate of 1.0 mL/min.

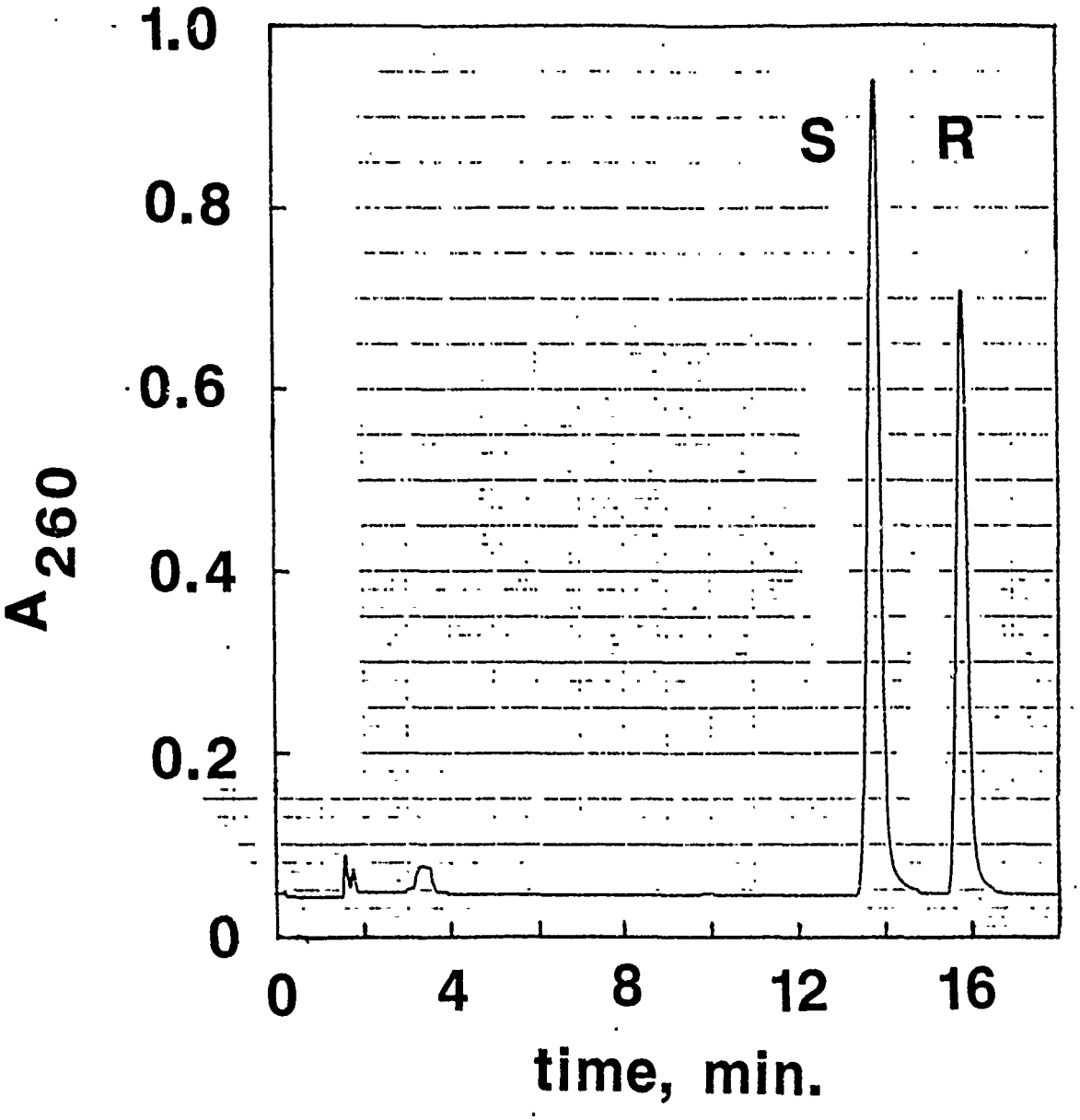


Fig. 3. Liquid chromatography of the T-T methylphosphonate pool from Fig. 2 into putative R and S diastereomers on a 4.6 x 250 mm column of $C_{18}$ silica, eluted with a gradient of 10-15% acetonitrile in water, 0.25%/min., at a flow rate of 1.0 mL/min.

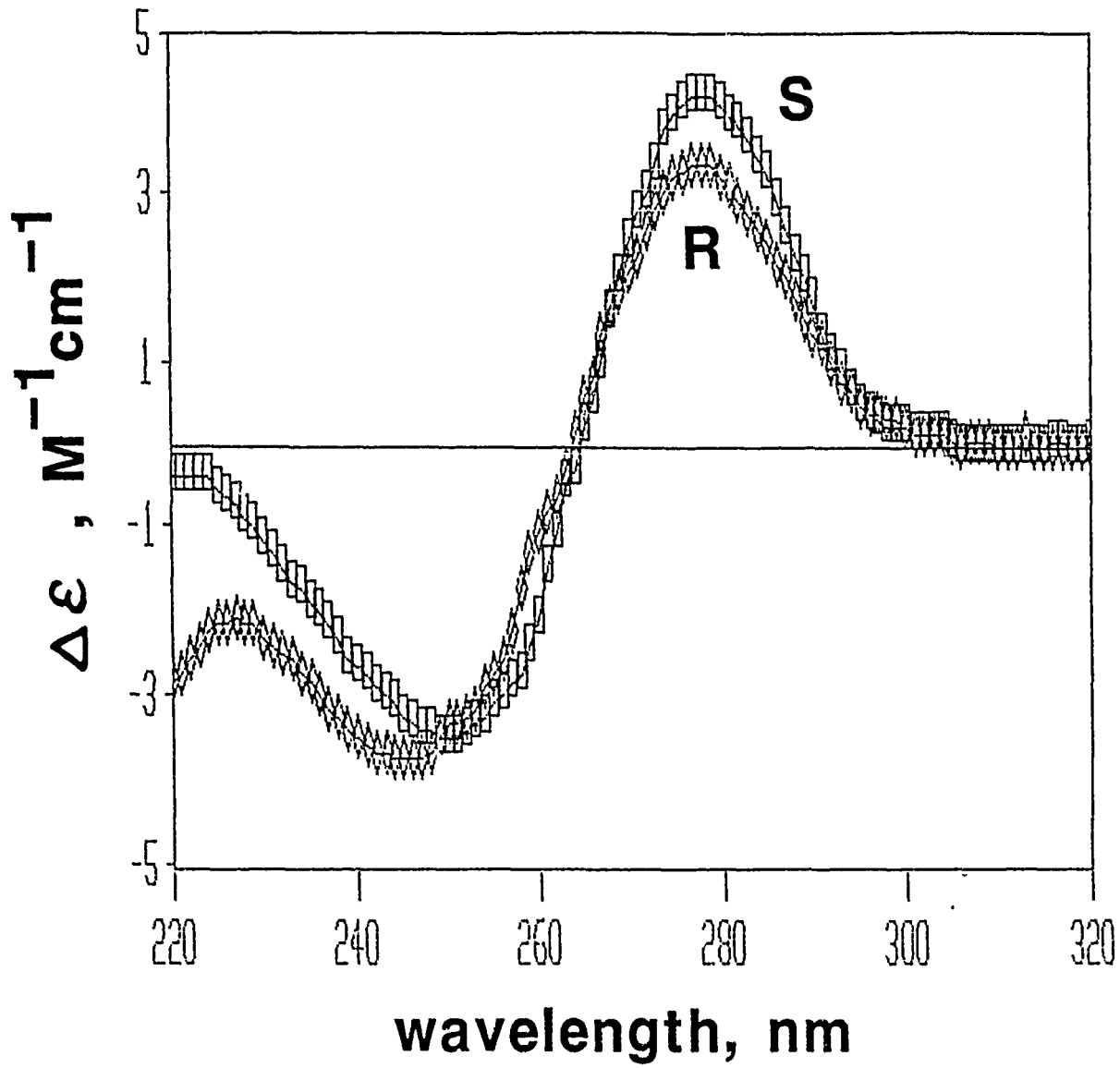


Fig. 4. Circular dichroism spectra of putative R and S diastereomers of T-T methylphosphonate separated in Fig. 3, in water at 25°, measured on the Jasco J-500A spectropolarimeter, USF.

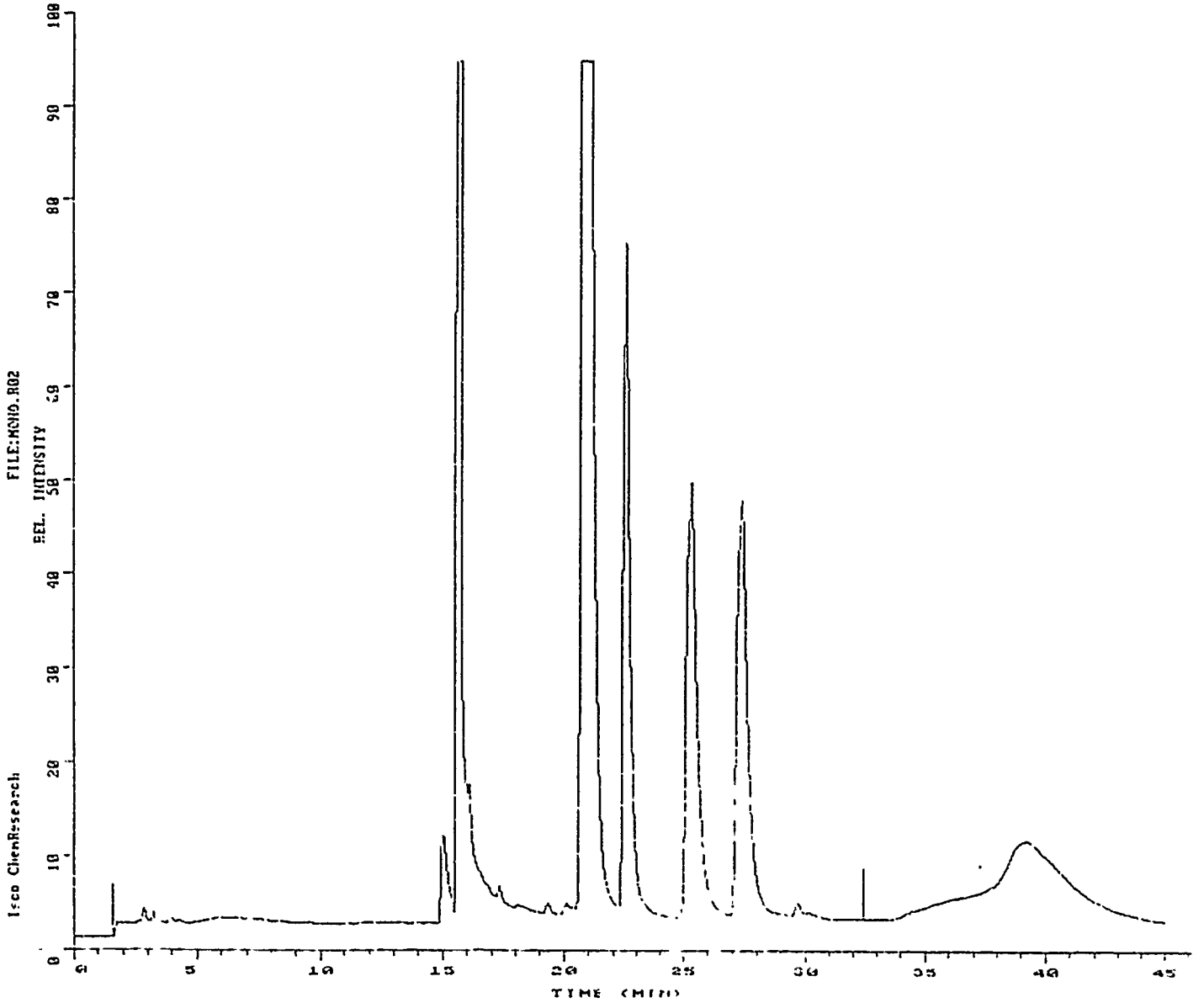


Fig. 5. Liquid chromatography of diastereomers of DMT-I-methylphosphonyl-I-Ac on a 10 x 250 mm column of silica eluted with 100% $CHCl_3$ for 5 min., followed by a gradient from 100% $CHCl_3$ to 20% MeOH, 80% $CHCl_3$ over 25 min., followed by a 10 min. segment up to 100% MeOH, at a flow rate of 5.0 mL/min. The peaks at 25.5 and 27.5 min. are the bis(inosine) methylphosphonate diastereomers.

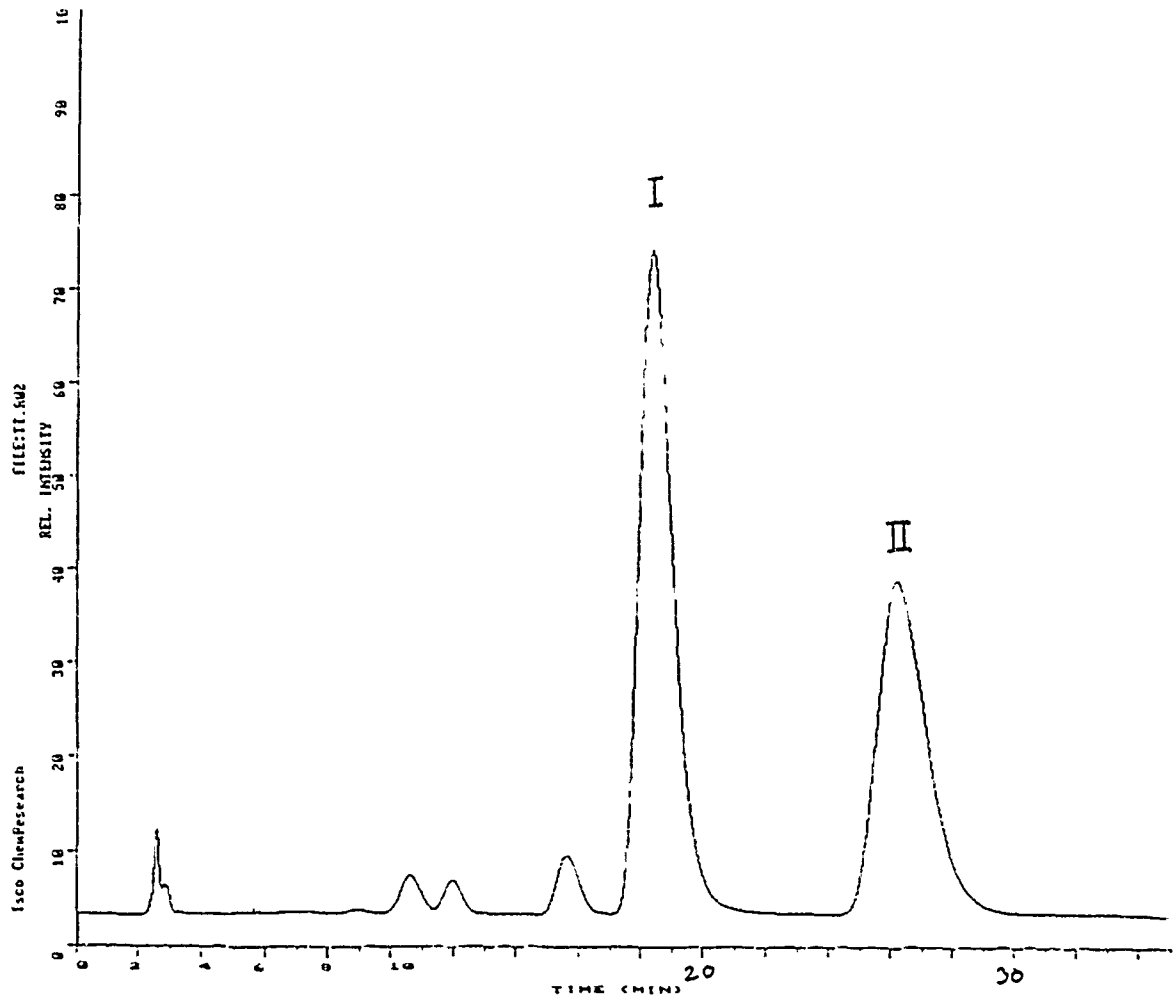


Fig. 6. Liquid chromatography of diastereomers of DMT-T-methylphosphonyl-T-Ac on a 10 x 250 mm column of silica eluted with 2% MeOH/98% $CHCl_3$ at a flow rate of 4.0 mL/min.

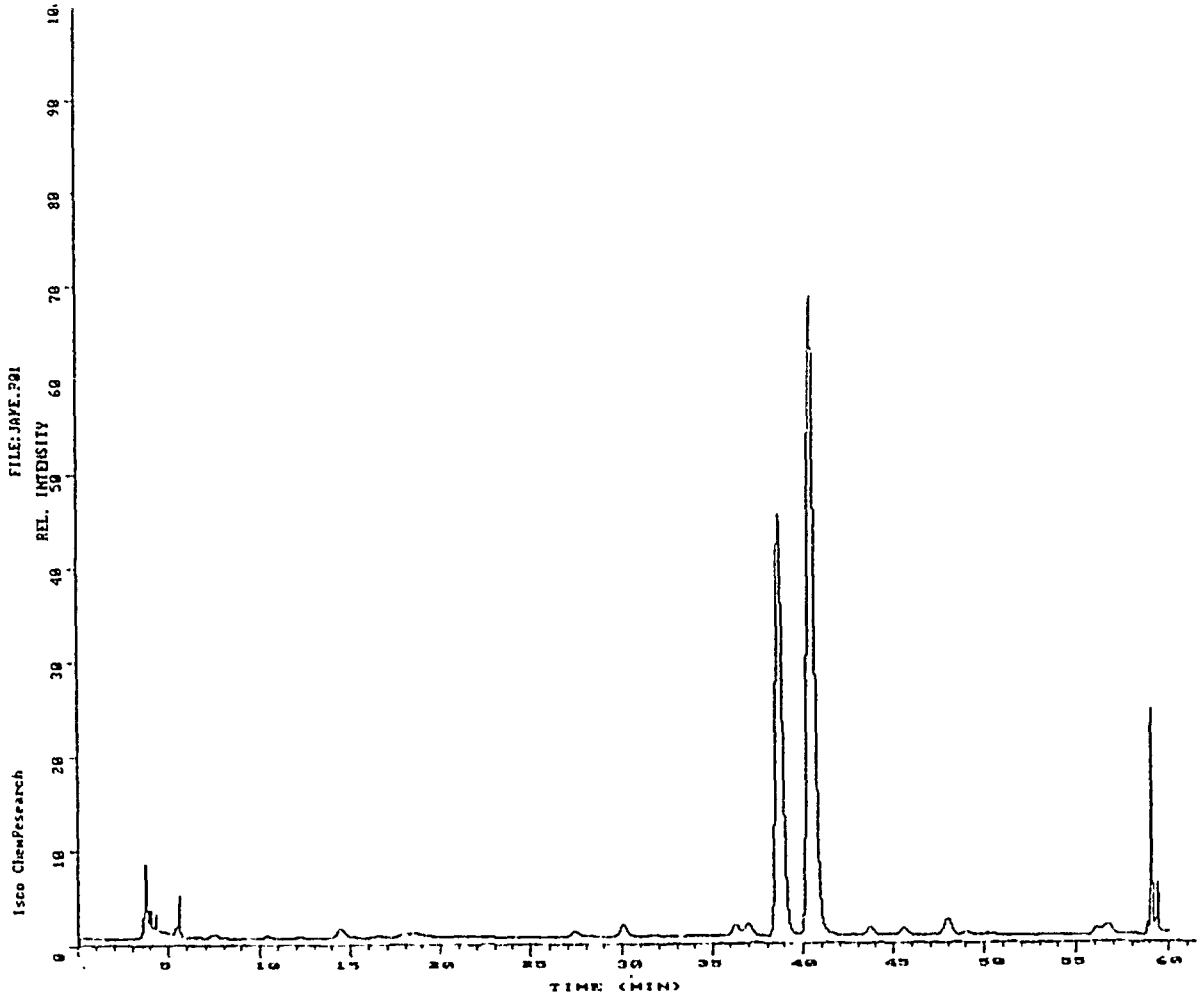


Fig. 7. Liquid chromatography of tetrathymidine methylphosphonate-3'-OAc, stereospecific at the first and third bonds, racemic at the middle bond, on silica, eluted with 2% MeOH in $CHCl_3$ for 15 min., followed by a gradient of 2-10% MeOH in $CHCl_3$ from 15 to 60 min. Y-axis recorded $A_{260}$, 0-2.0.

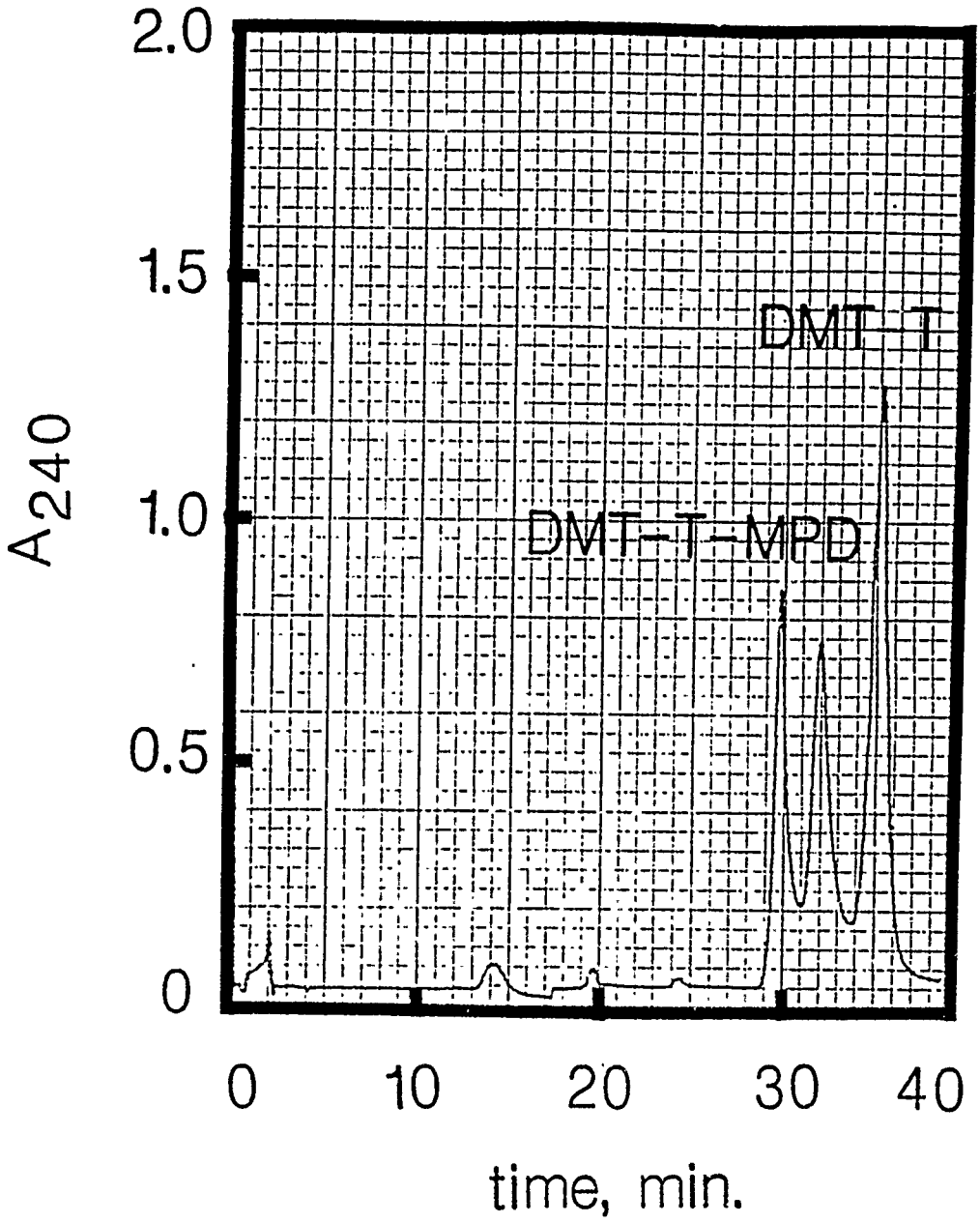


Fig. 8. Liquid chromatography of 5'-dimethoxytrityl thymidine 3'-methylphosphinyl diisopropylamidite on a 4.6 x 250 mm column of $C_{18}$ silica, eluted with 42% acetonitrile in water, at a flow rate of 1.0 mL/min. DMT-thymidine elutes at 35 min., and the peaks at 30 and 32 min. are the putative diastereomers of the methylphosphinyl diisopropylamidites.

5. *Oligodeoxynucleoside Methylphosphonate FABMS*

An initial test of FABMS on oligodeoxynucleoside methylphosphonates was carried out on racemic thymidinyl methylphosphonyl thymidine from Fig. 2. Shown in Fig. 9 are the fragment ions corresponding to various cleavages in the molecule which were seen in the low resolution FAB mass spectrum. In addition to the protonated molecular ion at m/z 545, we see the sequence ions resulting from cleavage between ribose and oxygen of the phosphate ester at m/z 321 and 225. A small ion at m/z 419 is also present and corresponds to fragmentation of one thymidine base with charge retention on the remainder of the molecule. All of these cleavage patterns have been seen in previous studies of alkylphosphotriesters (Phillips, et al., 1985). Similar clear results were seen for bis(deoxyinosine) methylphosphonate (Fig. 10).

The triethylammonium salt of a partially protected tetramer (5'CUAA3') was dissolved in LiCl, mixed with a mixture of glycerol and thioglycerol, and 0.5 $\mu$L corresponding to 175 ng was subjected to FABMS. A single scan spectrum was recorded in the positive ion mode and the resulting molecular ion and the structure for this tetramer is shown in Fig. 11.

The preparation of protected tetrathymidine methylphosphonate described in 2. above was analyzed similarly, yielding the mass spectrum in Fig. 12. Small peaks for the parent ion and tetramer fragments may be seen.

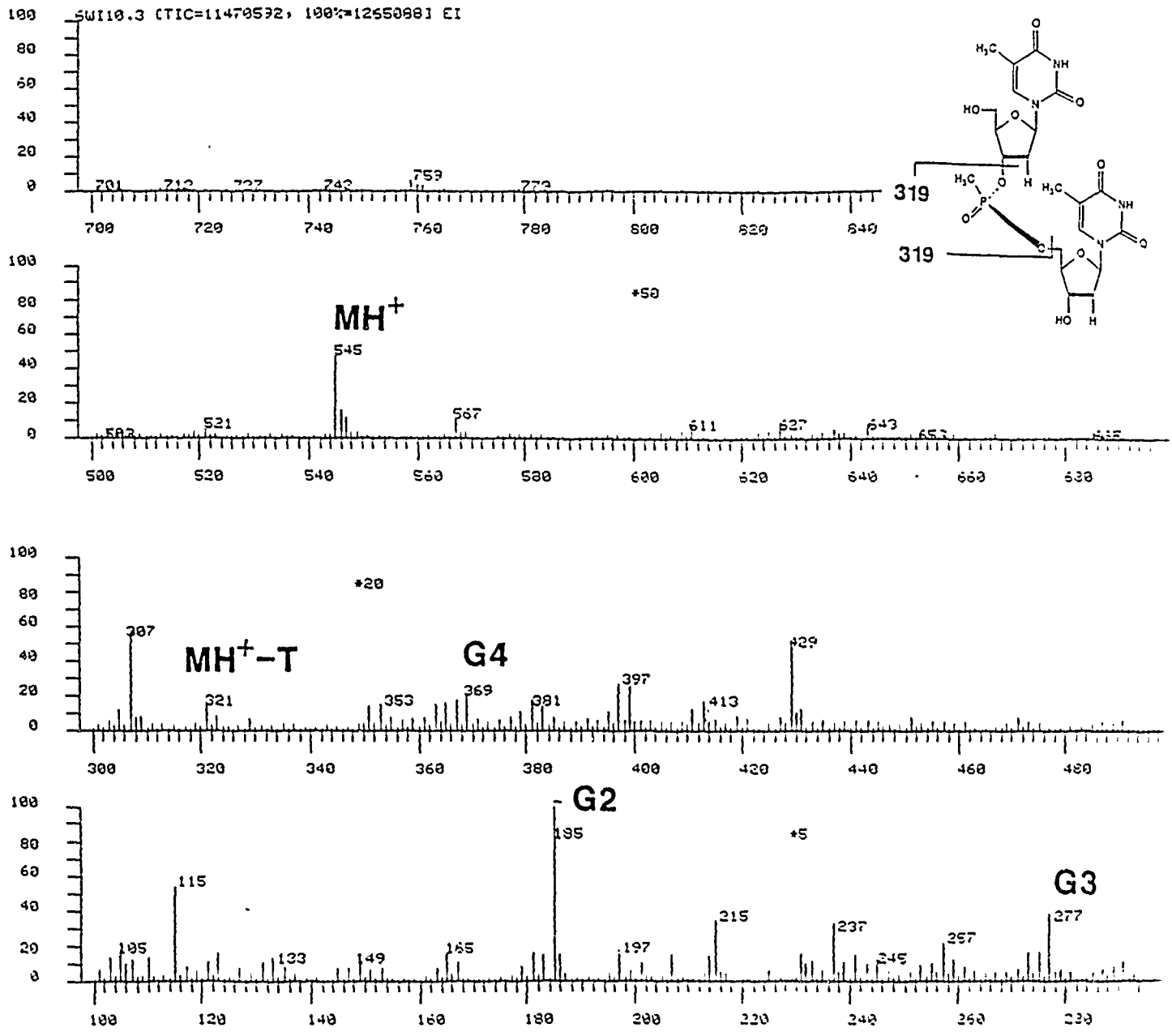


Fig. 9. Fast atom bombardment mass spectroscopy of racemic T-T methylphosphonate, sputtered from glycerol, on the Kratos MS 50 of Dr. Leary, Berkeley.

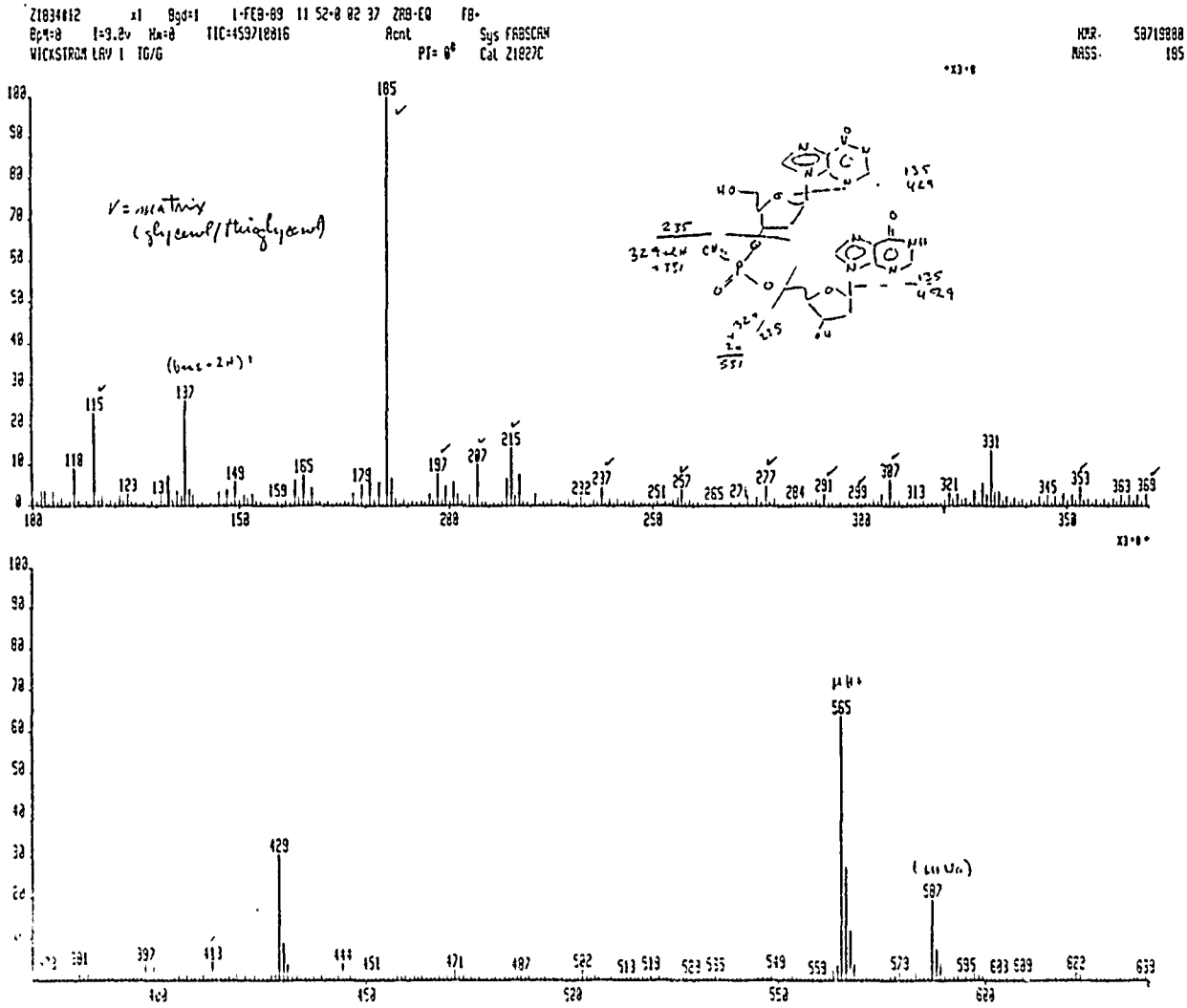


Fig. 10. Fast atom bombardment mass spectroscopy of stereospecific I-I methylphosphonate, sputtered from glycerol, on the Kratos MS 50 of Dr. Leary, Berkeley.

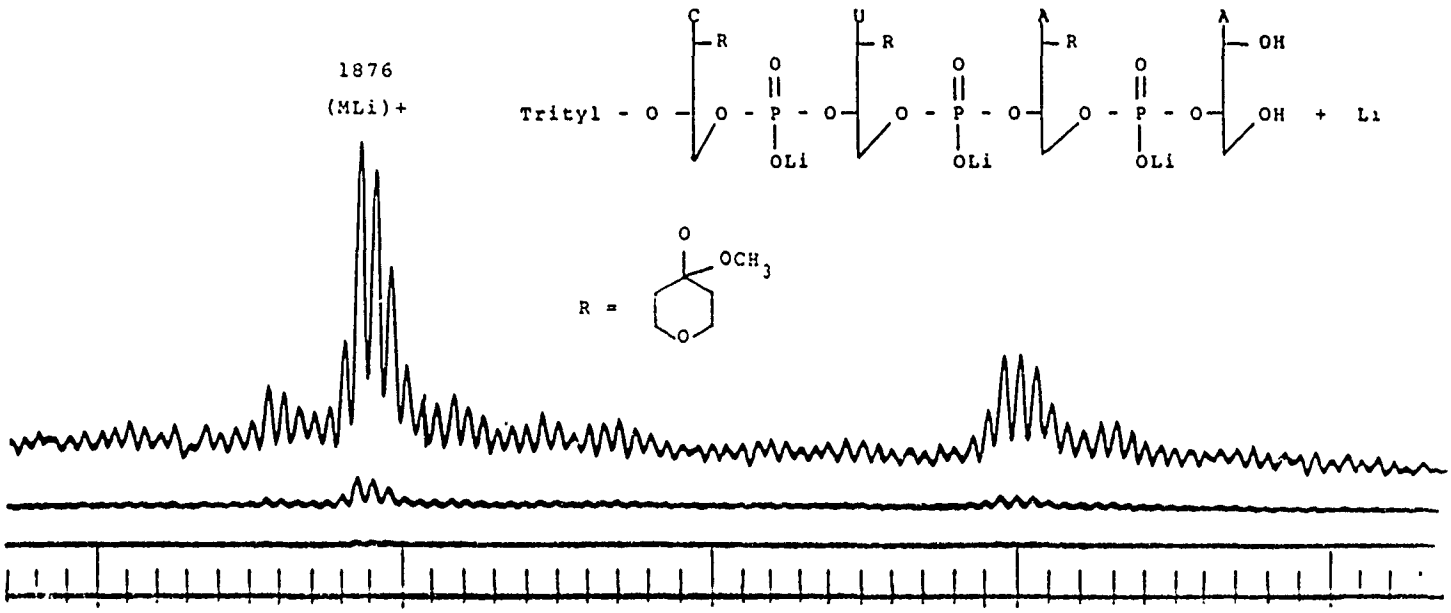


Fig. 11. Fast atom bombardment mass spectroscopy of the lithium salt of 5'-trityl-CUAA, sputtered from a mixture of glycerol and thioglycerol, on the Kratos MS 50 of Dr. Leary, Berkeley.

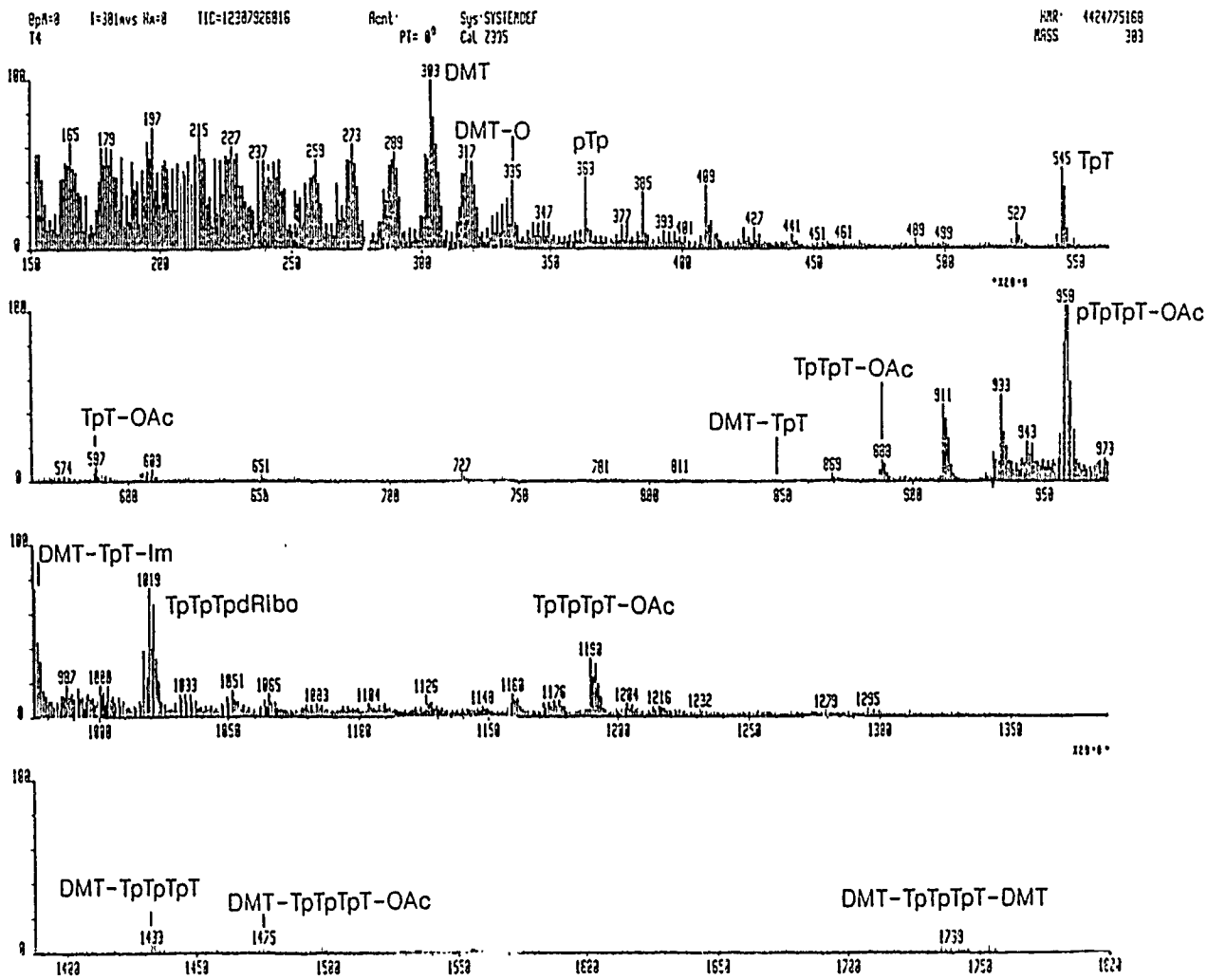


Fig. 12. FABMS of 5'-DMT-tetrathymidine methylphosphonate-3'-OAc, sputtered from thioglycerol, on the instrument of Dr. Leary, Berkeley.

*6. Oligodeoxynucleoside Methylphosphonate NMR*

One-dimensional spectra have been measured for a series of intermediates and dimers. The activated monomer DMT-thymidine methylphosphonyl imidazolide gave expected $^{1}H$ and $^{31}P$ spectra (Fig. 13), as did the separated diastereomers of thymidine methylphosphonyl thymidine (Figs. 14,15). The expected $^{31}P$ spectra were also obtained for the protected diastereomers of DMT-thymidine methylphosphonyl thymidine-acetate (Fig. 16).

For the trivalent route, 5'-dimethoxytrityl thymidine methylphosphinyl diisopropylamide coupled very rapidly to 3'-O-benzoyl thymidine, as measured by $^{31}P$ NMR (Fig. 17). The expected $^{31}P$ spectra were also obtained for 5'-dimethoxytrityl thymidine methylphosphonyl diisopropylamidite reacting with 5'-activated 3'-O-acetyl thymidine in the **absence** of tetrazole (Fig. 18). Tetrazole was eliminated expressly to prevent epimerization, and its catalytic role was substituted by a proprietary 5' activation of 3'-O-acetyl thymidine.

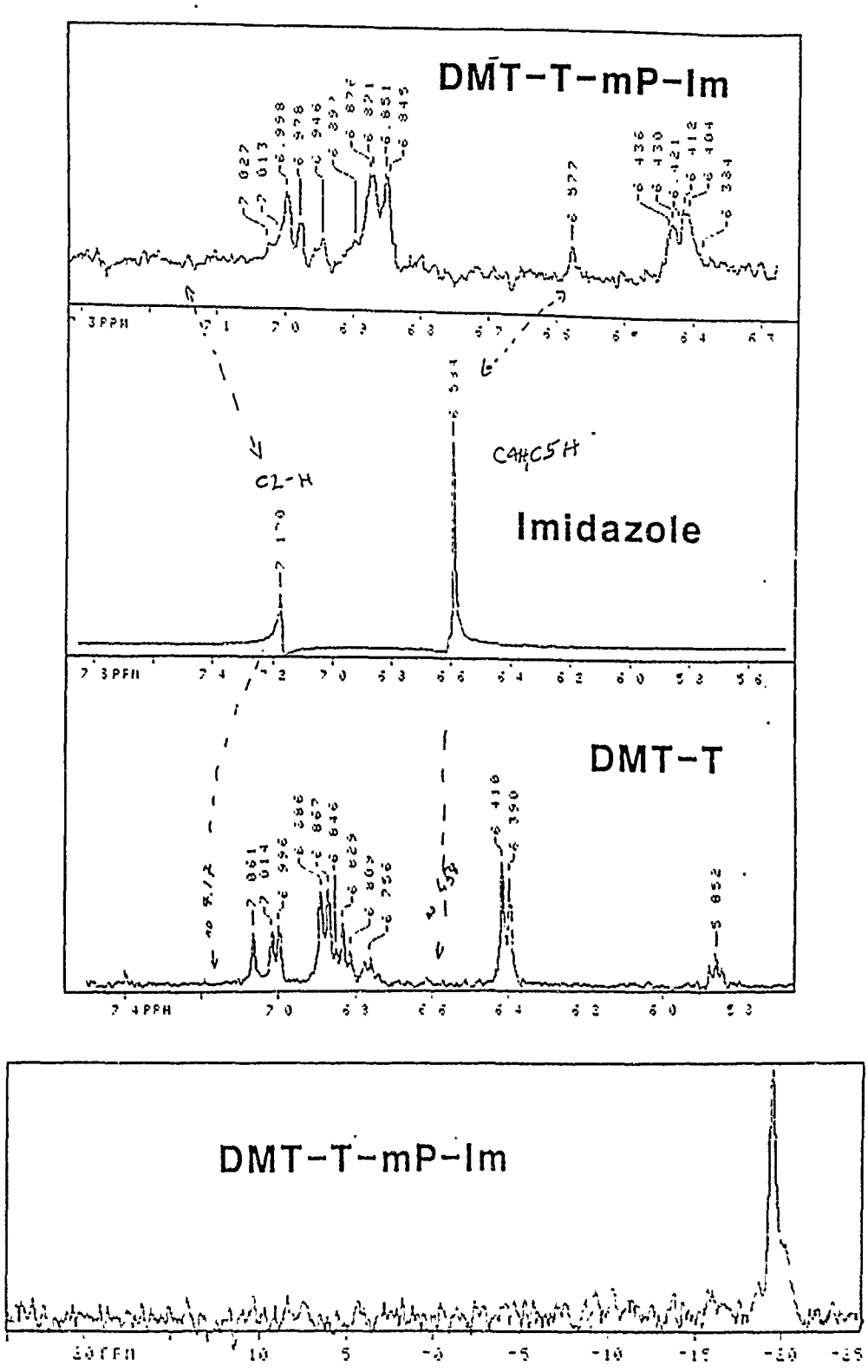


Fig. 13. $^{1}H$ and $^{31}P$ NMR of DMT-thymidine methylphosphonyl imidazolide peak from Fig. 1, measured on the Varian 400 MHz of Dr. Cohen, NCI.

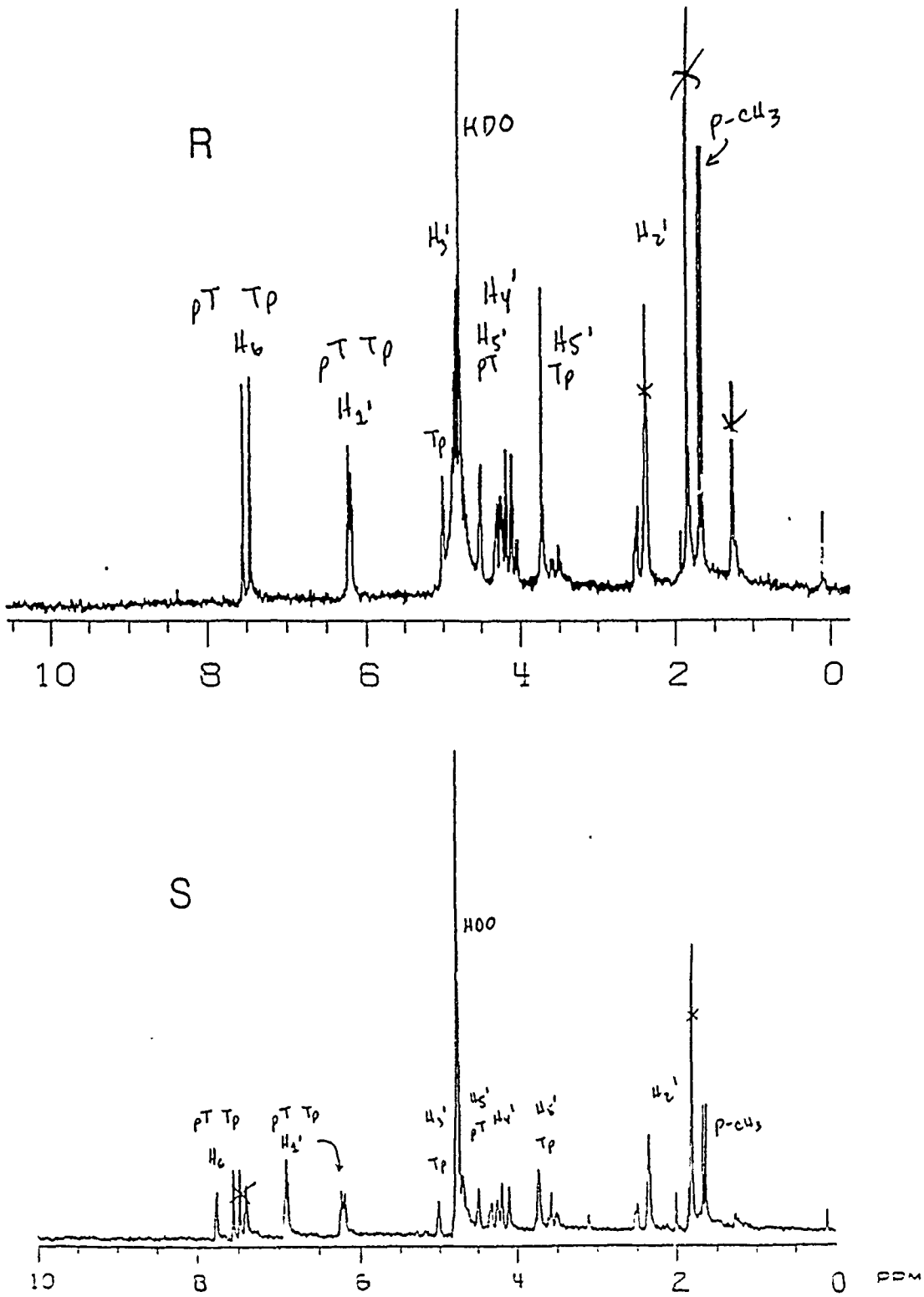


Fig. 14. $^{1}H$ NMR of putative R and S diastereomers of thymidine methylphosphonyl thymidine, in $^{2}H_2O$, measured on the NT 470 of Dr. Gorenstein, Purdue.

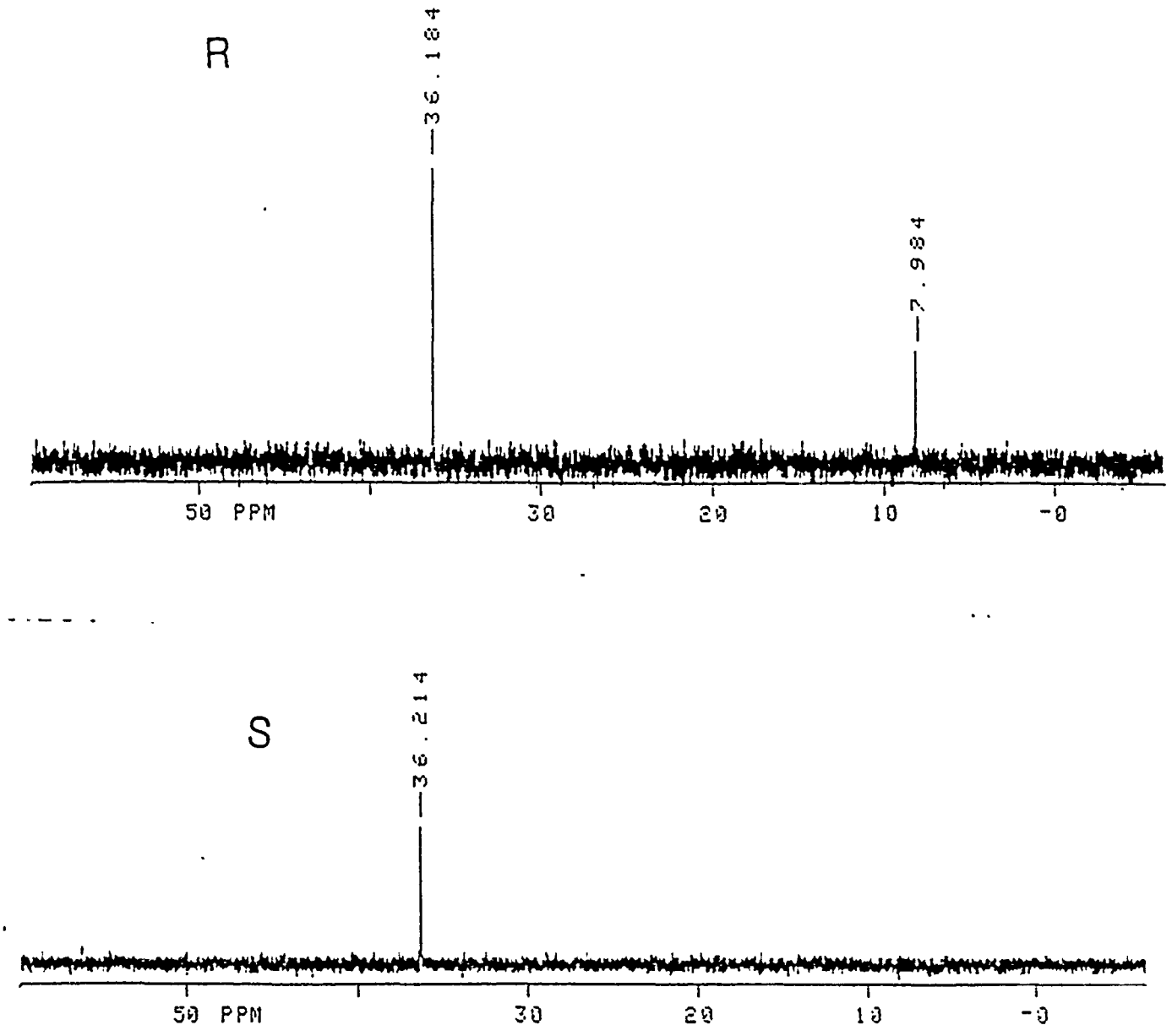


Fig. 15. $^{31}P$ NMR of putative R and S diastereomers of thymidine methylphosphonyl thymidine, in $^{2}H_2O$, relative to phosphoric acid, measured on the XL 200 of Dr. Gorenstein, Purdue.

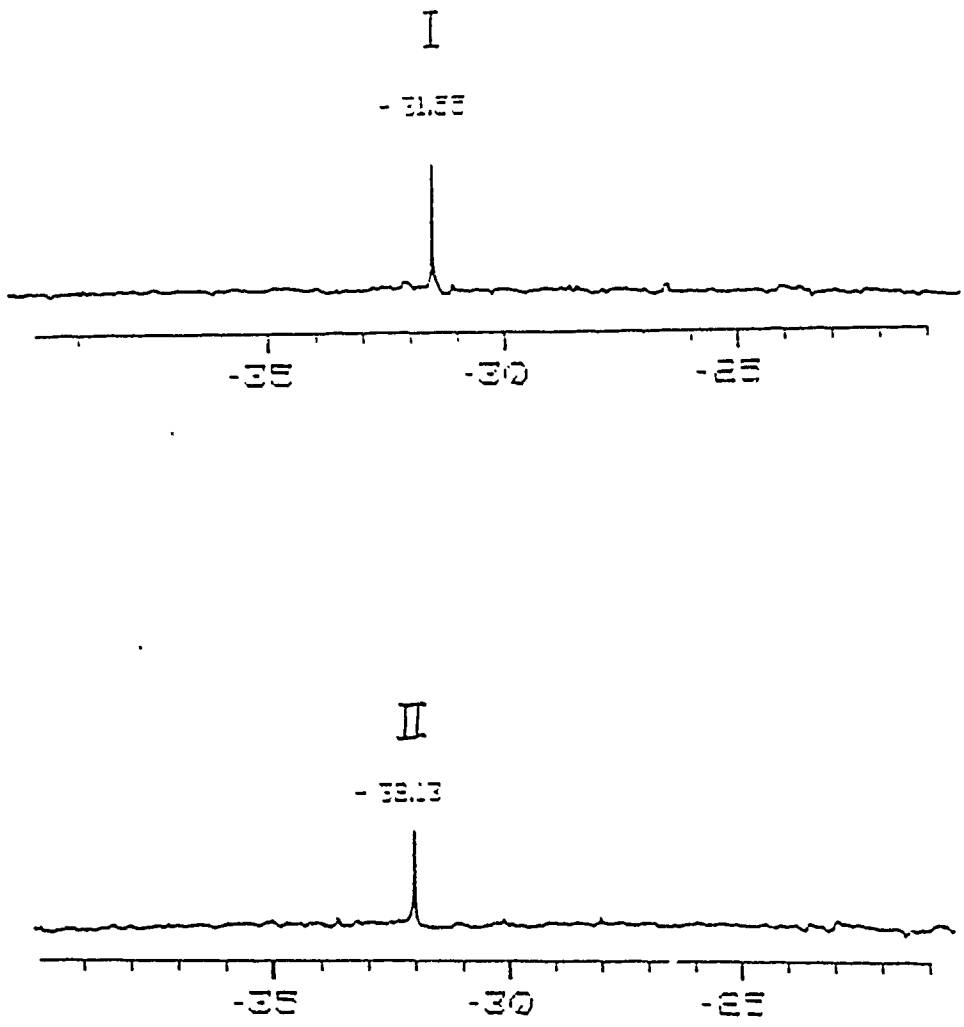


Fig. 16. $^{31}P$ NMR of DMT-T-methylphosphonyl-T-Ac peaks from Fig. 5, measured on the Jeol FX90Q, USF.

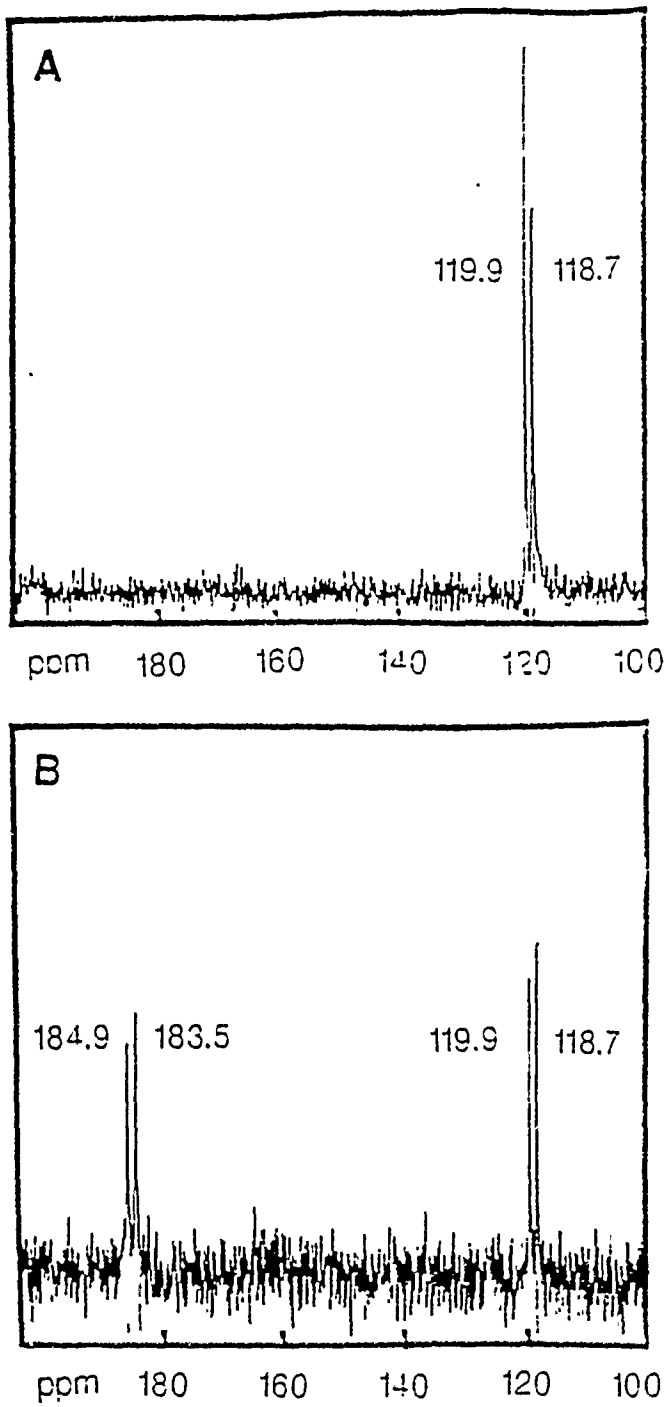


Fig. 17. $^{31}P$ NMR of excess DMT-T methylphosphinyl diisopropylamidite and limiting 3'-O-benzoyl thymidine, in $C^2H_3CN$, relative to phosphoric acid, measured on the JEOL FX90Q, USF. A, prior to adding tetrazole; B, 8 min. after adding an equivalent of tetrazole.

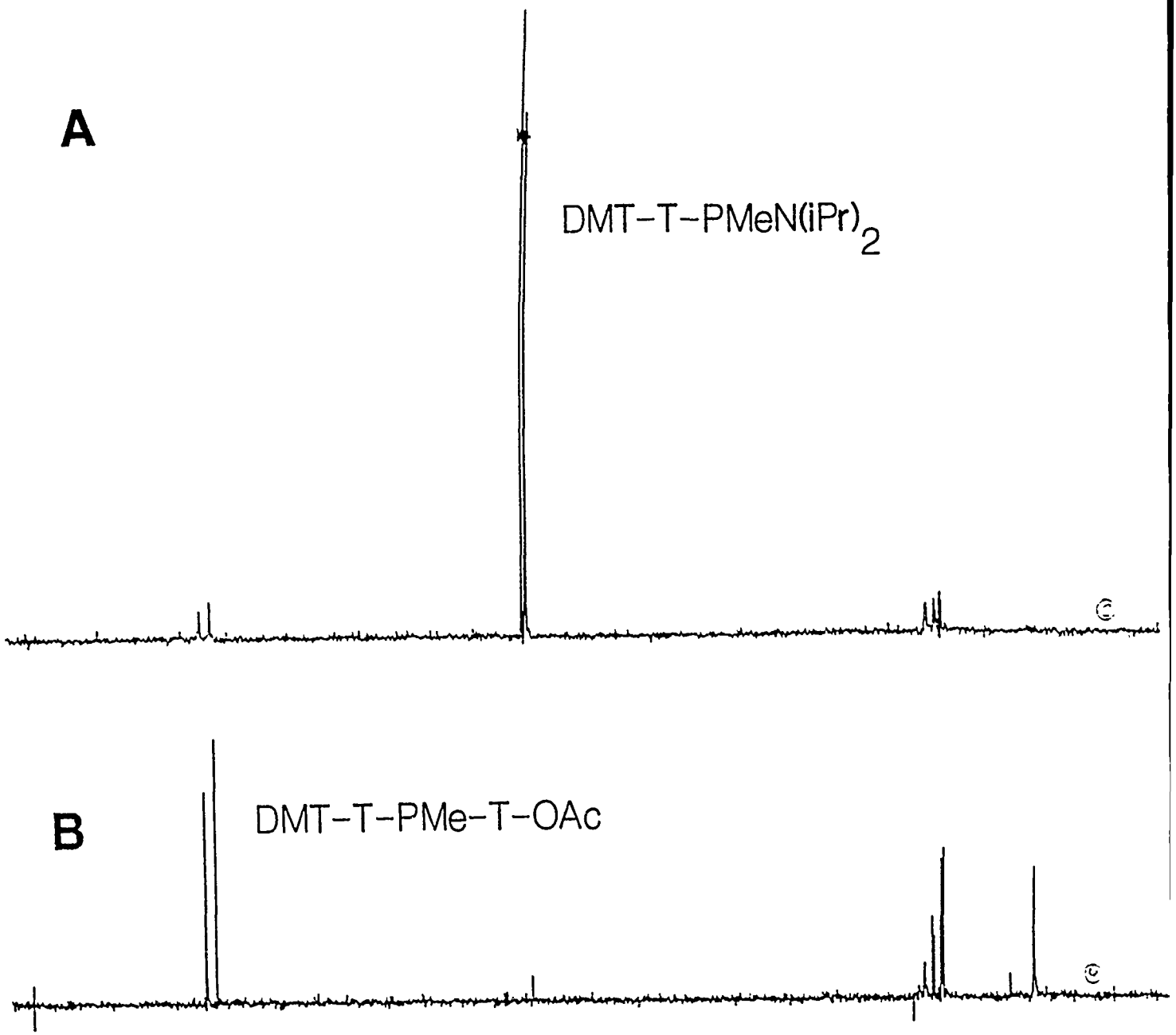


Fig. 18. $^{31}P$ NMR of equimolar DMT-T methylphosphonyl diisopropylamidite and 5'-activated 3'-O-acetyl thymidine, in $C^2H_3CN$, relative to phosphoric acid, measured on the JEOL FX90Q, USF. A, shortly after mixing; B, at the end of the reaction.

*7. Antisense Oligodeoxynucleotide Inhibition of HIV Gene Expression*

Prediction of the secondary structure of the known sequence of the *tat* gene placed the initiation codon near the 3' end of a large loop (Fig. 19), so an antisense oligodeoxynucleotide, 5'-dCATTTCTTGCTCTCC, was prepared against nt 5399-5414 of the HIV sequence (Sodroski, et al., 1985). Human T4 cells immortalized by HTLV-I are attacked and killed by HIV in vitro; protection against the HIV cytopathic effect is a useful screening technique for candidate therapeutics (Mitsuya and Broder, 1986). Our anti-*tat* pentadecamer is currently being tested for its ability to protect immortalized T4 cells against HIV cytopathic effect in the laboratory of Dr. Samuel Broder, Clinical Oncology Program, at NCI. The initial positive results showed large statistical variations, but gave no false positives for a control non-AIDS oligodeoxynucleotide, and no false negatives for cells plus oligodeoxynucleotide but without added virus (Fig. 20). However, addition of oligodeoxynucleotide to clumped ATH8 cells gave no protection against HIV virus, as opposed to the positive results obtained when the cells were in suspension when exposed to oligodeoxynucleotide. Two further trials gave more equivocal results than the first trial, with less significant protection of cells against viral challenge, but in any case no false positives were seen in any trial with control antisense sequences.

Fig. 19 predicts further single stranded targets between the 5' end and the initiation codon, including a hairpin loop in the TAR sequence (TAT1), and another loop 80 nt downstream (TAT3). The latter two sequences, as well as the 5' end (TAT0) and the initiation codon AUG (TAT9) were utilized as targets for antisense inhibition. The anti-*tat* oligomers were assayed in the 3B9 indicator cell line which constitutively expresses *tat*, and expresses $\beta$-galactosidase under *tat*-dependent HIV-LTR control (Bacheler, et al., 1989) in the laboratory of Dr. Lee Bacheler (Fig. 21), and in H9 cells transfected with pHXB2gpt (Fisher, et al., 1986) by Dr. David Looney in the laboratory of Dr. Flossie Wong-Staal (Fig. 22). In both cases, TAT1 and TAT3 displayed significant inhibition of HIV expression at 50 $\mu$M and above, but not TAT0 or TAT9.

Secondary structure calculations have also been carried out on the *gag-pol* (Fig. 23) and *env* mRNAs (Fig. 24) in order to predict single stranded targets in those messengers. Antisense oligomer hybridization arrest will also be attempted at the newly predicted targets.

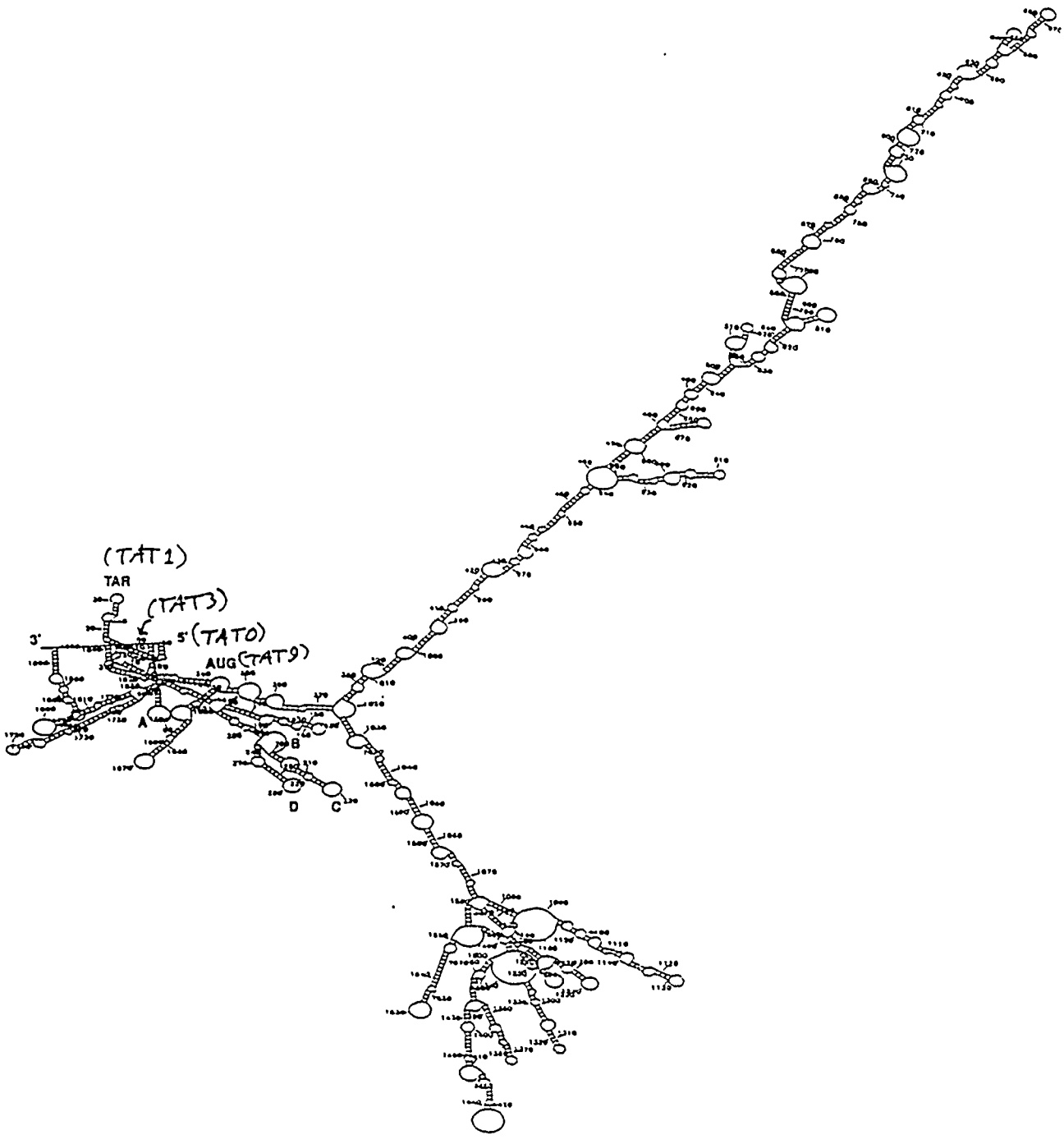


Fig. 19. Predicted secondary structure of the entire HIV *tat* mRNA. TAR indicates the transactivation responsive sequence, AUG indicates the initiation codon, and letters indicate potential targets.

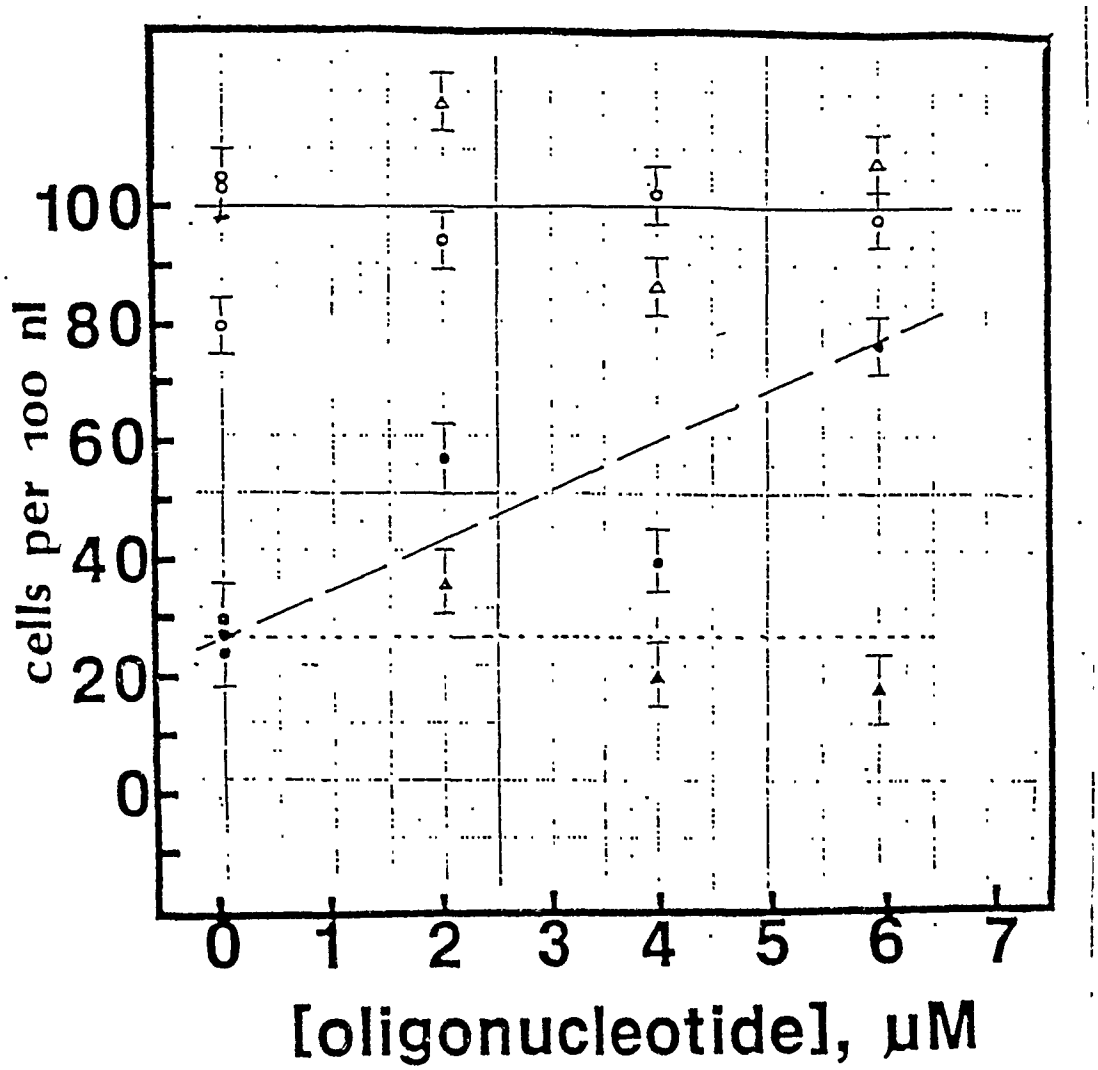


Fig. 20. Antisense oligodeoxynucleotide inhibition of HIV cytopathic effect on ATH8 cells. In each assay (Mitsuya and Broder, 1986), 2 x $10^5$ cells were resuspended in 1 mL RPMI 1640 with 11.5% fetal calf serum supplemented with recombinant interleukin 2, and added to sterile vials containing lyophilized oligodeoxynucleotide. Cells were challenged with HIV at 500 virions/cell after a 1 hr. incubation period, then grown for 9 days at 37° in 5% $CO_2$. Titers of viable cells were determined by counting Trypan blue excluding cells in a hemocytometer. Circles represent protection by 5'-dCATTTCTTGCTCTCC, complementary to HIV nt 5399-5414, while triangles represent protection by 5'-dTTGGGATAACACTTA, complementary to VSV M gene nt 17-31. Filled symbols represent cells challenged by HIV, while open symbols represent cells not challenged by HIV, showing the lack of toxicity of the oligodeoxynucleotides. The experiments were carried out by Dr. Makoto Matsukura in the laboratory of Dr. Samuel Broder, National Cancer Institute.

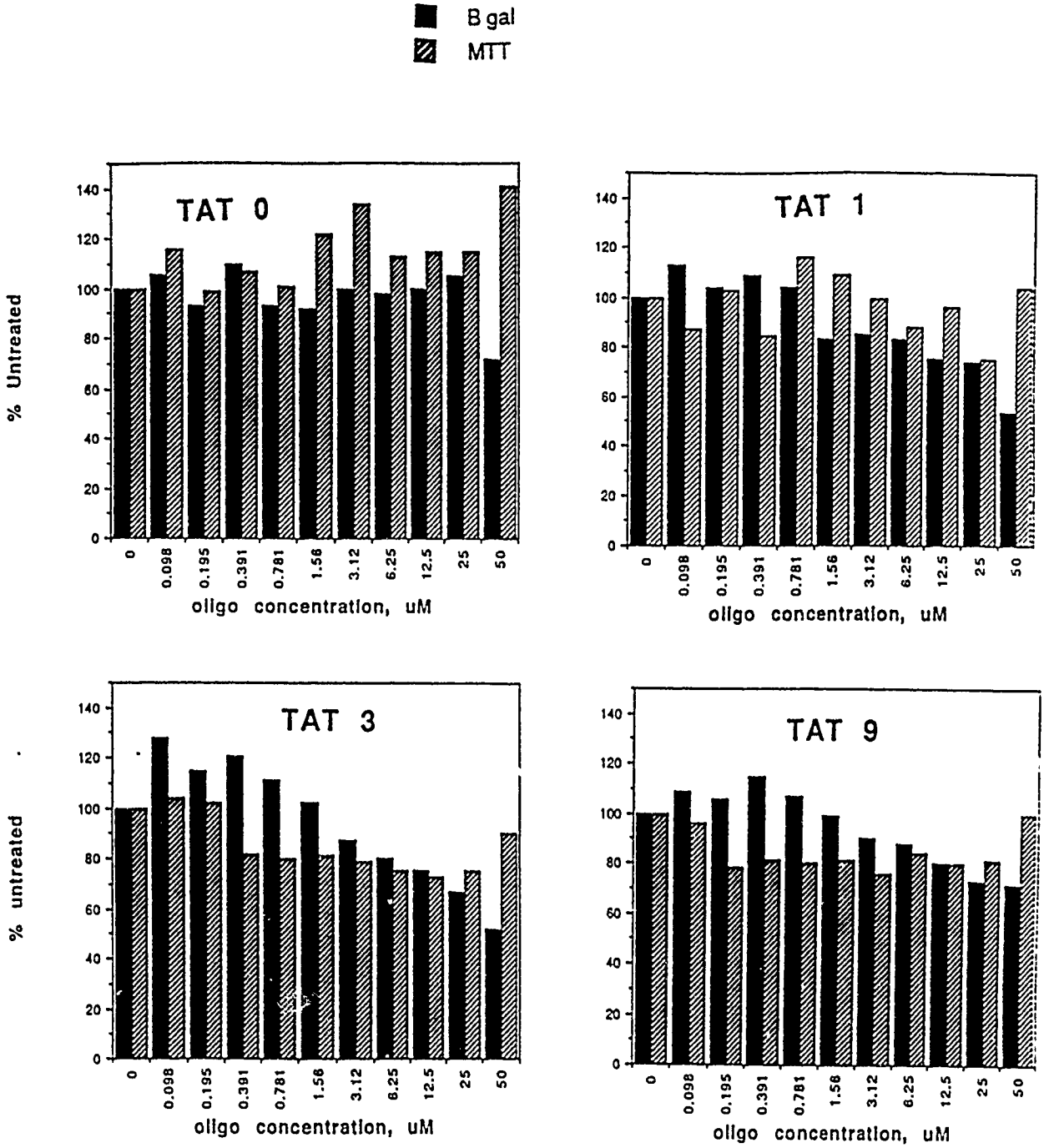


Fig. 21. Antisense inhibition of *tat*-dependent β-galactosidase expression in 3B9 indicator cells (Bacheler, et al., 1989). 3B9 cells were seeded at 16,000 cells per 100 μL well in DMEM with 2% LTSR (Sigma), a serum-free medium. After a day of recovery at 37° in 5% $CO_2$, the medium was replaced with fresh medium containing oligodeoxynucleotide at the concentrations shown, and incubated for another 24 hr. Cells were then assayed for β-galactosidase activity, and cell viability by MTT tetrazolium dye uptake. TAT0: 5'-end; TAT1: TAR loop; TAT3: nt 104-118; TAT9: AUG loop.

| Conc. ($\mu$M) | TAT0 IFA/MNGC | TAT1 IFA/MNGC | TAT3 IFA/MNGC | TAT9 IFA/MNGC | Control IFA/MNGC |
|---|---|---|---|---|---|
| 100.0 | +/+ | -/- | -/- | +/+ | +/+ |
| 50.0 | +/+ | -/- | -/- | +/+ | +/+ |
| 25.0 | +/+ | +/+ | +/+ | +/+ | +/+ |
| 12.5 | +/+ | +/+ | +/+ | +/+ | +/+ |
| 6.3 | +/+ | +/+ | +/+ | +/+ | +/+ |
| 3.2 | +/+ | +/+ | +/+ | +/+ | +/+ |
| 1.6 | +/+ | +/+ | +/+ | +/+ | +/+ |
| 0.0 | -/- | -/- | -/- | -/- | -/- |

Fig. 22. Antisense oligodeoxynucleotide inhibition of HIV p24 expression and formation of syncytia. In each assay 4 x $10^4$ SupT1 cells were added to each well and incubated for 1 hour at 37 degrees C, after which 10 x TCID-50 of a preparation of the HIV-1 molecular clone pHXB2D was added to each well. Wells were fed at 48 hours with 100 $\mu$l of media containing corresponding concentrations of oligomers freshly prepared from 1 mM stock solutions in PBS. Oligomers TAT0, TAT1, TAT3, and TAT9 are the same sequences as in Fig. 2. Oligomers and PBS control were diluted 1:5 in RPMI 1640 media with 20% heat-inactivated fetal bovine serum, 1% Penicillin-Streptomycin, and 2 mM L-glutamine for use. Duplicate parallel dilutions were made into a final volume of 100 $\mu$l yielding concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0 $\mu$M. The 0 $\mu$M sample was *not* challenged with the viral clone. After 4 days of incubation, each well was inspected for the presence of multi-nucleated giant cells (MNGC), and cells from each well were fixed and stained for HIV-1 p24 by indirect immunofluorescent microscopy (IFA).

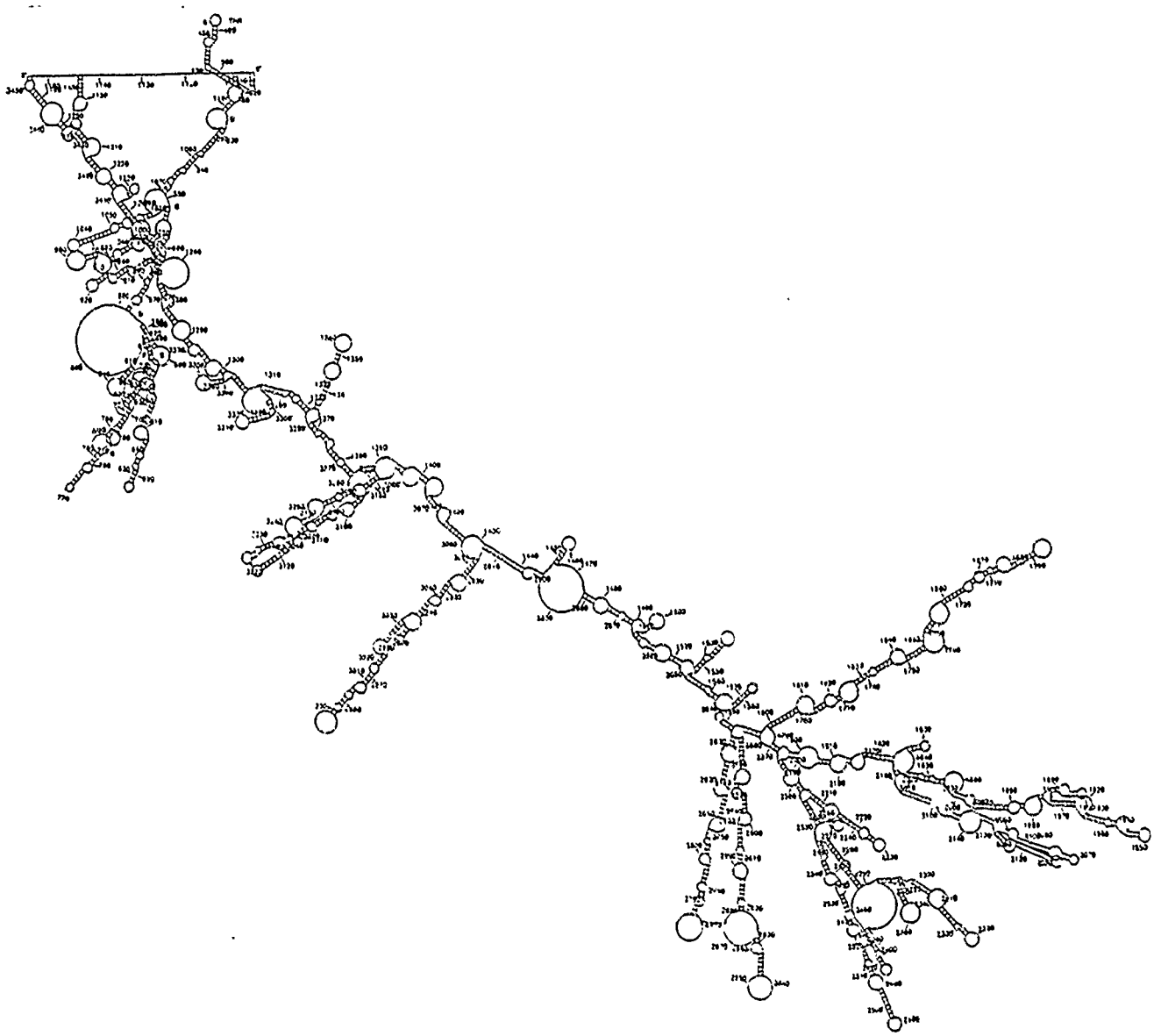

Fig. 23. Predicted secondary structure of the first 3,000 nt of HIV *gag-pol* mRNA, as in Fig. 19.

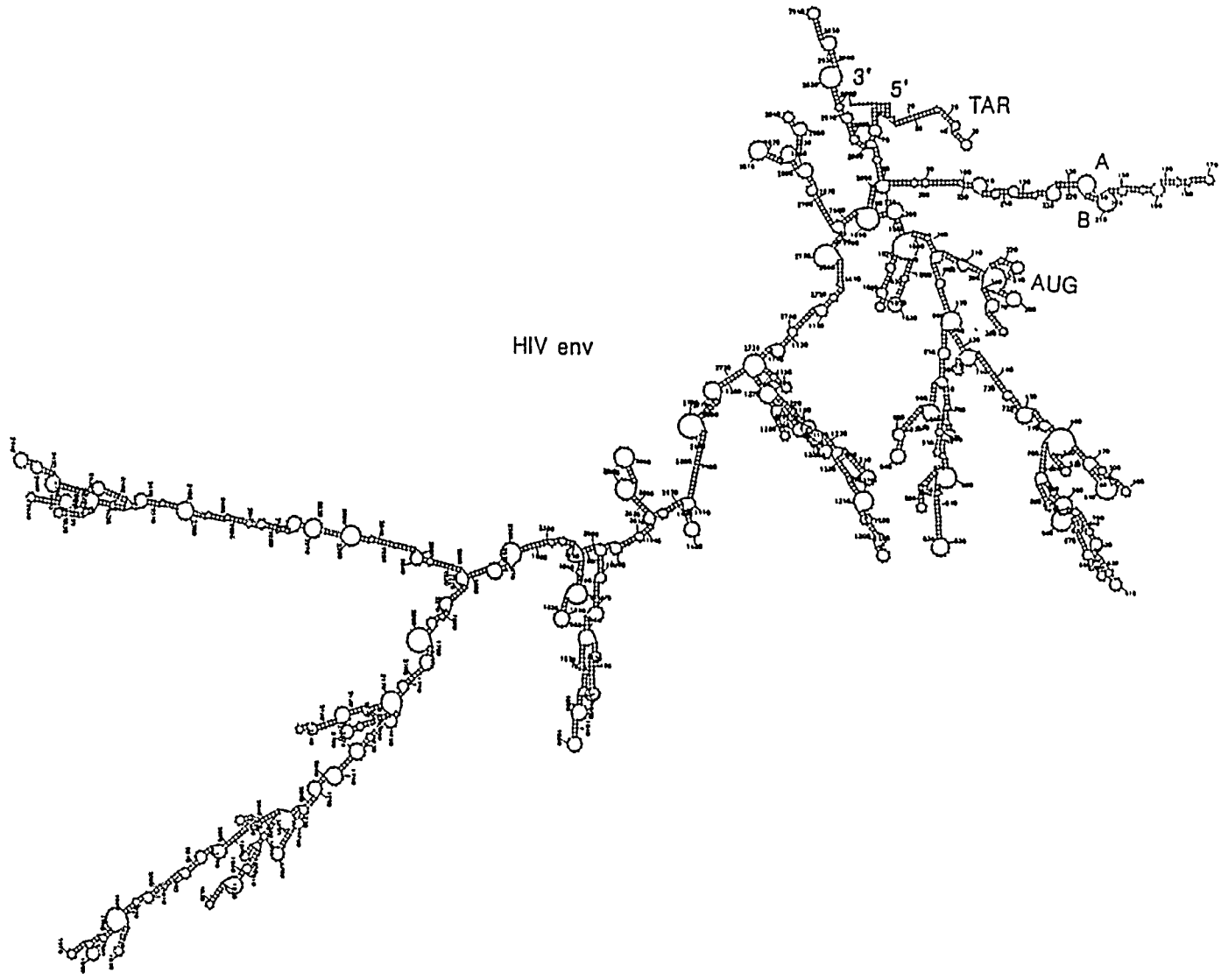


Fig. 24. Predicted secondary structure of the first 3,000 nt of HIV *env* mRNA, as in Fig. 19.

8. *Optimum Targets for Antisense Oligomer Hybrid Arrest within an mRNA* (Preprint in Appendix)

Recently, a series of different sequences complementary to predicted loops, bulges, and helices between the cap and initiation codon of human c-*myc* mRNA have been synthesized. HL-60 cells in culture were treated for 24 hr. with 10 $\mu$M of each oligomer, plus controls. The cells were then metabolically labelled with [$^{35}$S]Met, and c-*myc* p65 antigen was analyzed by radioimmunoprecipitation. It was apparent that the 5' cap was an even better target than the initiation codon bulge, but that the region in between presented poor targets for hybrid arrest. At the original target, a 12-mer was not as efficacious as the original 15-mer, and an 18-mer was more effective. However, the differences were not dramatic, as a simple thermodynamic model would have predicted. We suspect that we are observing the effects of both RNase H attack, at all locations, and genuine hybrid arrest, at the sensitive cap and AUG sites.

9. *c-myc Transgenic Mice*

In order to take advantage of their nuclease resistance and greater uptake by cells, racemic oligodeoxynucleoside phosphorothioates and methylphosphonates have been synthesized, and the latter displayed about half the efficacy of normal oligodeoxynucleotides for antiproliferation of HL-60 cells (not shown). Following experiments with cells in culture, the nuclease resistant antisense oligodeoxynucleoside methylphosphonates will be tested for efficacy in transgenic mice carrying the HIV genome. Parallel studies are already being done in transgenic mice carrying the c-*myc* gene under the control of an immunoglobulin heavy chain enhancer (Adams, et al., 1985). This work is being done in collaboration with Drs. Ralph Brinster and Richard Palmiter, with the particular help of Dr. Brinster's postdoctoral fellow, Dr. Eric Sandgren, who recreated the line for these experiments. Similar work will be done on HIV transgenic mice, in collaboration with Dr. Gilbert Jay, NCI.

The c-*myc* transgenic mice typically die by the age of 4 months of highly malignant, disseminated B-cell and pre-B-cell lymphomas with associated leukemia. We wish to see whether or not antisense oligodeoxynucleoside methylphosphonate therapy can prevent or inhibit transgenically induced B-cell lymphoma in an animal model. As a first step, we have found that we can detect overexpressed human c-*myc* p65 antigen in the nuclei of transgenic lymphocytes by IFA, following shipment of frozen buffy coats from Philadelphia to Tampa (not shown). This experiment was done twice, with negative controls, single blind at our end.

The next step was 3 hr. of treatment with anti-c-*myc* oligomer, delivered by tail vein injection to about 150 $\mu$M, which successfully suppressed human p65 expression (Fig. 25). Injection of a scrambled sequence, or saline, had no effect. At a lower dose, 25 $\mu$M, no inhibition was detected. These experiments were done double blind, in Philadelphia and in Tampa. We have also determined the levels of serum oligodeoxynucleoside methylphosphonates at 3 min. after injection, and at 1 and 3 hr. by reversed phase liquid chromatography of acetonitrile-deproteinized serum supernatants (Fig. 26). The oligomer showed no sign of degradation, and the serum levels dropped rapidly over the first hr., but appeared to level out thereafter (Fig. 27). Perhaps the oligodeoxynucleoside methylphosphonates partition rapidly throughout body tissues, which then provide a reservoir to maintain serum levels. Excretion by the kidneys has not yet been determined.

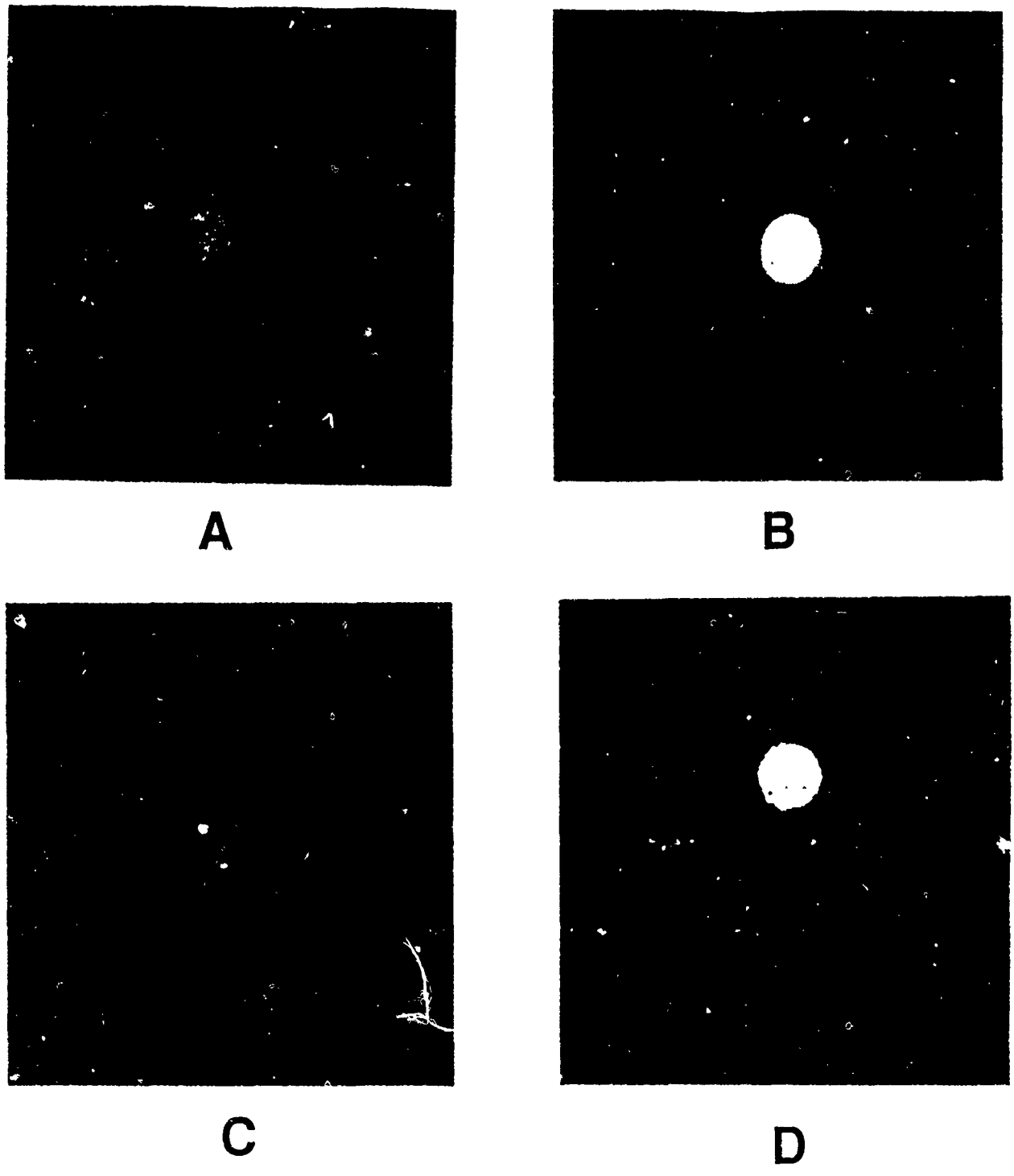


Fig. 25. Indirect immunofluorescence of leukocytes from human c-*myc* transgenic mice, injected with 300 nmol oligodeoxynucleoside methylphosphonate in 250 $\mu$L PBS. Aliquots of 250 $\mu$L of blood were removed at 3 hr. Leukocytes were isolated, then fixed with 1% paraformaldehyde, and stained with rabbit polyclonal anti-c-*myc* IgG, followed by fluorescein-conjugated goat anti-rabbit IgG. A, normal mouse; B, transgenic mouse injected with saline; C, transgenic mouse injected with anti-c-*myc* oligomer; D, transgenic mouse injected with scrambled oligomer.

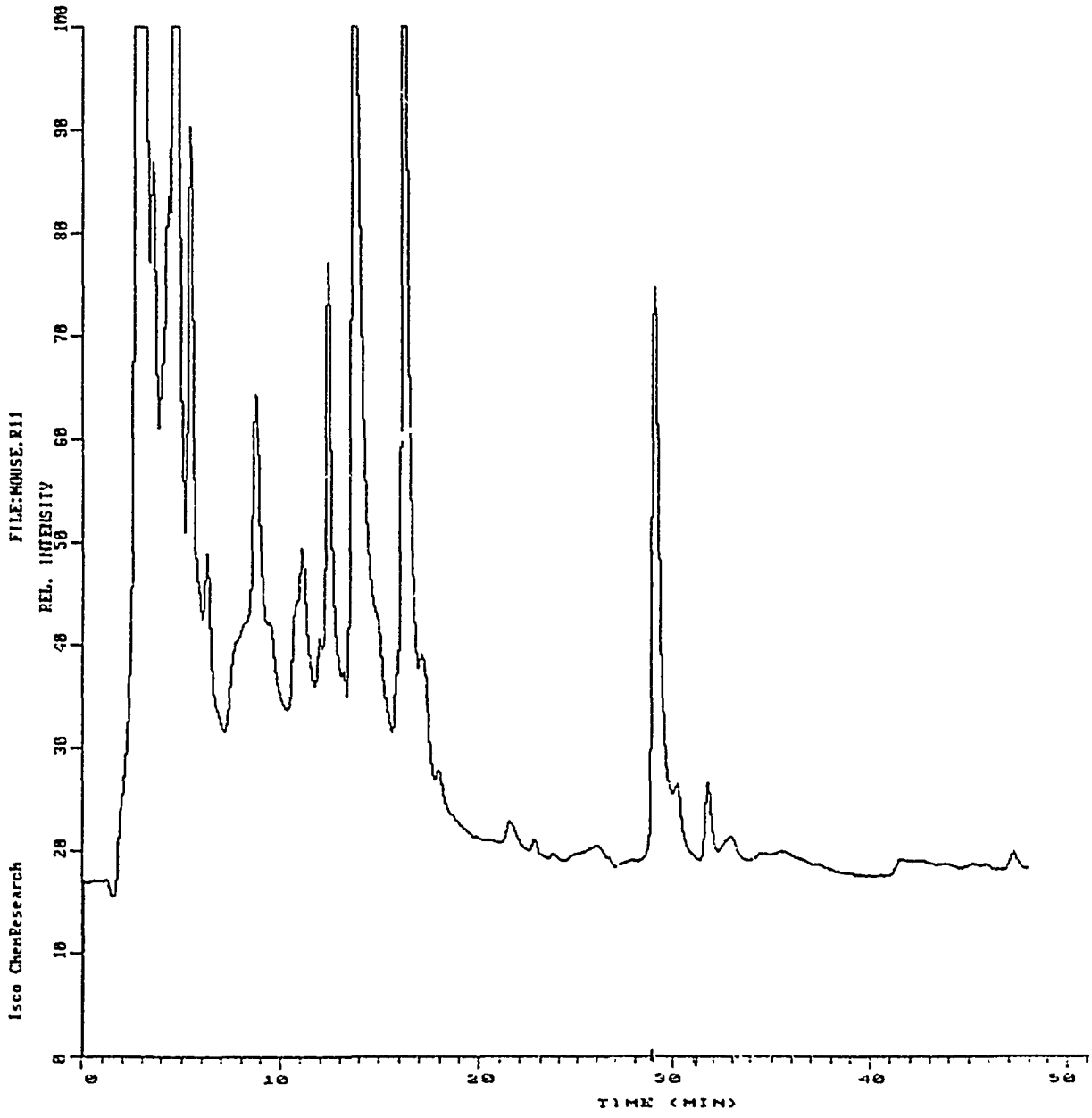


Fig. 26. Liquid chromatography of oligodeoxynucleoside methylphosphonates recovered from serum samples of an individual c-*myc* transgenic mouse. Aliquots of 250 $\mu$L of blood were removed at 3 min., 1 hr., and 3 hr. An equal volume of $CH_3CN$ was added to each sample, and insoluble material was removed by sedimentation. Each supernatant was passed through a 0.2 $\mu$m filter, evaporated, redissolved in water, and analyzed by liquid chromatography on a 4.6 x 250 mm column of $C_{18}$-silica 5 $\mu$m particles, eluted with a gradient from water to $CH_3CN$, and detected by absorbance at 260 nm Chromatogram shows the 1 hr. sample.

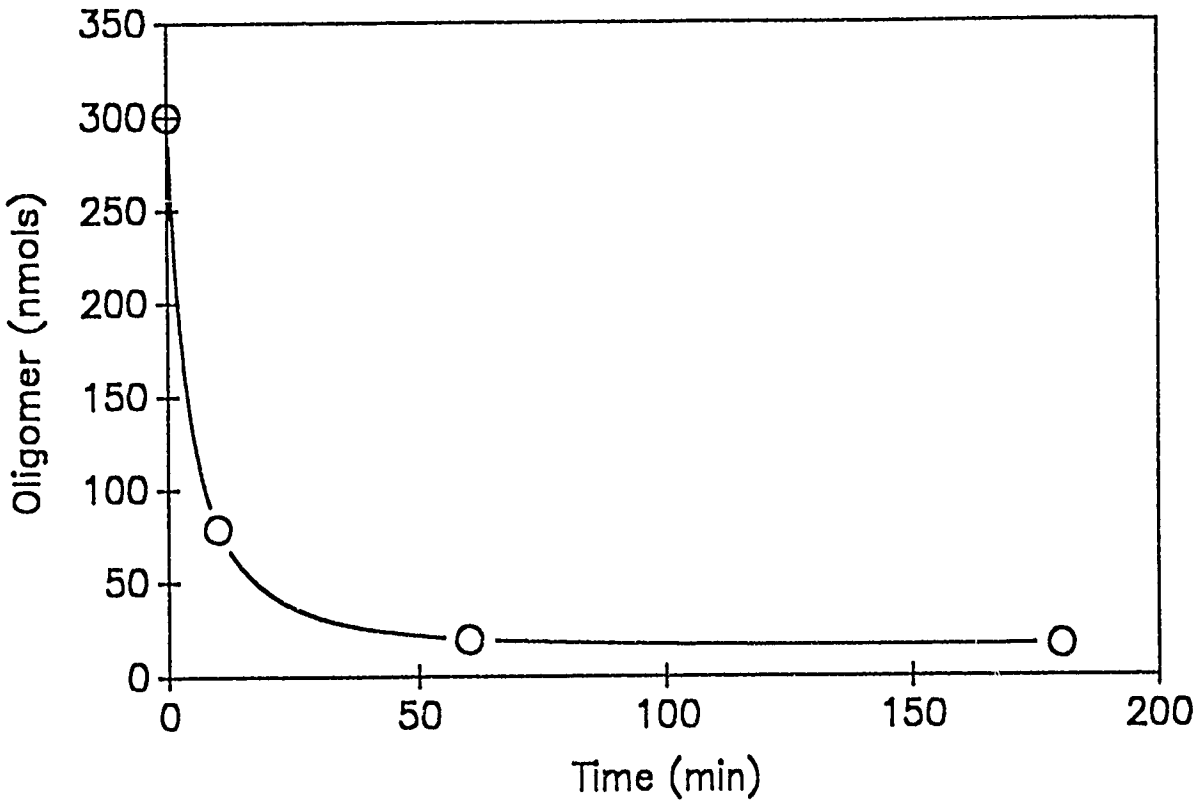


Fig. 27. Recovery of oligodeoxynucleoside methylphosphonates from serum samples of an individual c-*myc* transgenic mouse. Samples removed at 3 min., 1 hr., and 3 hr. were analyzed by liquid chromatography as in Fig. 14, and quantitated by integrated peak areas.

10. *MHC Class I Antigens*

We have received the cell line KE-2, which produces a broad spectrum anti-MHC I monoclonal antibody, and CV-1, which constitutively expresses MHC class I surface antigens, from Dr. Robert Ricciardi (Vasavada, et al., 1986). The KE-2 MAb immunoprecipitates a strong MHC I band from peripheral blood lymphocytes of a healthy donor, but very little from a similar number of HL-60 cells (Fig. 28). We are currently investigating whether anti-c-*myc* oligomers, which down-regulate c-*myc* expression, can in turn up-regulate MHC I expression in HL-60 cells. If this occurs, we will then determine whether or not the treated cells become more susceptible to cytotoxic T lymphocytes. Parallel experiments will then be done with HIV-transformed human cell lines.

As a sidelight to the question of immune surveillance, a collaboration was carried out with the laboratory of Dr. Louis Chedid on the relationship between peptide secondary structure and immunogenicity (Lise, et al., 1989) (reprint in Appendix). The peptides $(NANP)_4$ and $(NANP)_8$ mimic the 37-fold repeated NANP epitope of *Plasmodium falciparum* circumsporozoite protein, and are quite immunogenic; the altered sequences $(NANG)_4$ and $(NANG)_8$ are much less immunogenic. CD spectra of these sequences revealed that the immunogenic proline-containing wild type sequences were almost entirely random coil, while the poorly immunogenic glycine-containing mutant sequences displayed a small fraction of $\alpha$-helical character. Hence, immunogenicity correlated positively with a lack of secondary structure.

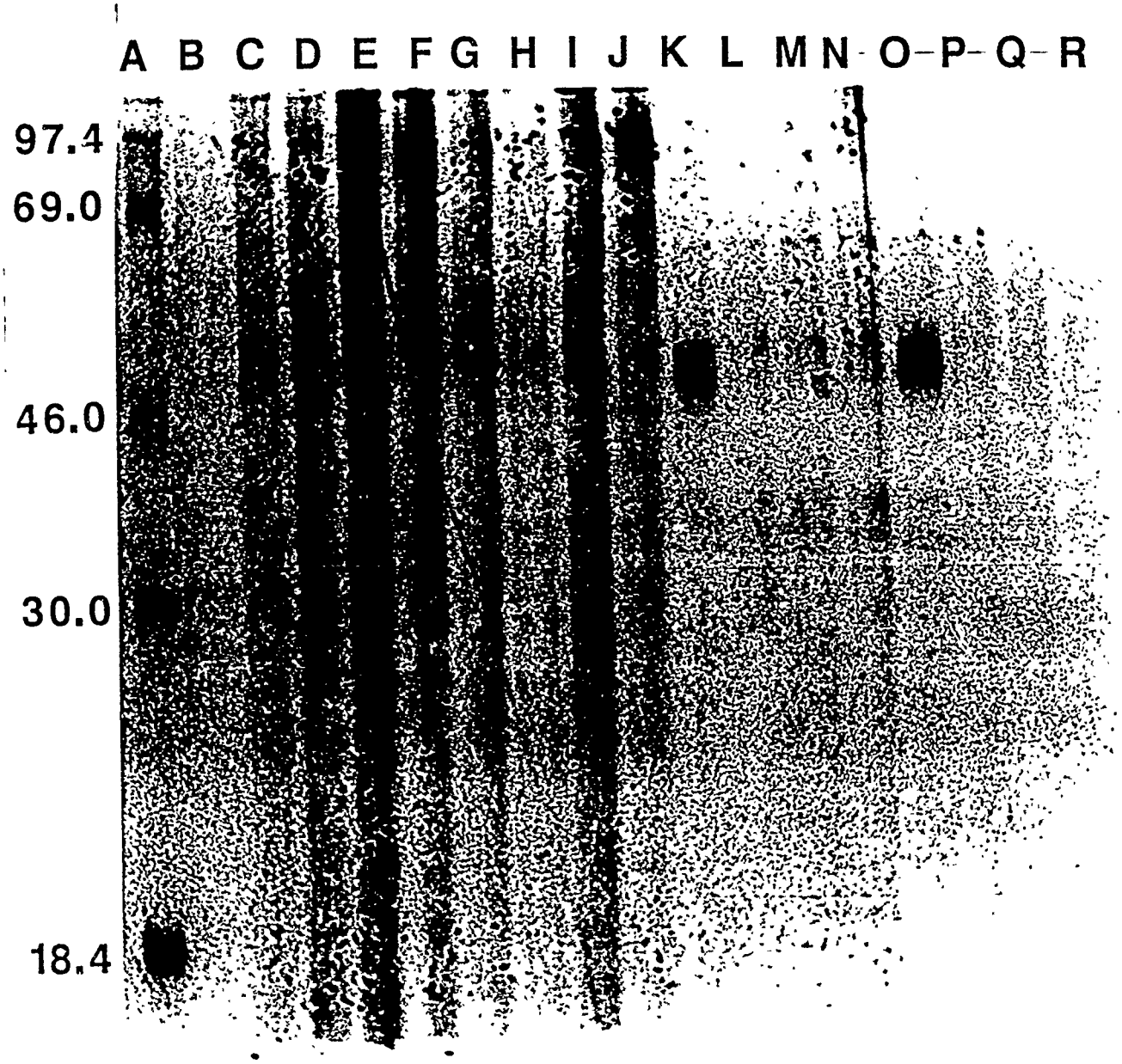


Fig. 28. Fluorogram of denaturing polyacrylamide gel of KE-2 MAb radioimmunoprecipitated MHC class I antigens from HL-60 cells (lanes C, serum-free, and G, with serum) or adult human lymphocytes (lanes K, PHA-stimulated, and O, non-stimulated) metabolically labelled for 5 hr.

11. *DNA Receptors*

Upon looking to see whether any of our cell lines displayed the putative DNA receptor proteins characterized by Bennett, et al. (1988), preliminary immunofluorescence studies of HL-60 cells with MAb 24T showed cell surface staining, with some apparent capping (Fig. 29). We would like to determine whether or not these antibodies inhibit oligodeoxynucleotide uptake, and interdict antisense oligodeoxynucleotide inhibition of c-*myc* expression. If positive results are found, we will then investigate whether or not oligodeoxynucleoside phosphorothioates or methylphosphonates are taken up by this receptor. Partial trypsinization will also provide a useful probe of the behavior of the putative receptor. A panel of transformed cell types, as described above for MHC I studies, will be analyzed next, along with unstimulated peripheral blood lymphocytes.

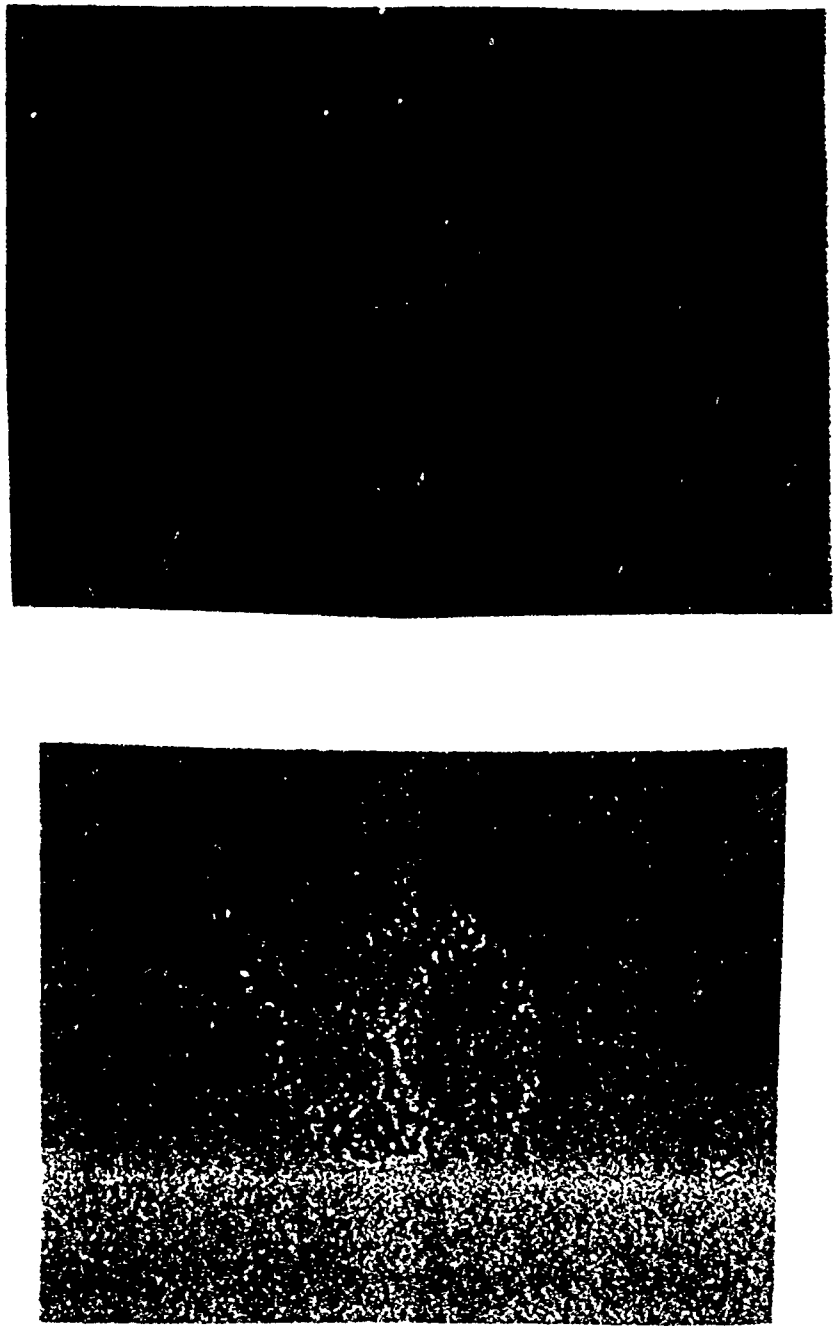

Fig. 29. Indirect immunofluorescence of HL-60 cells, stained with mouse monoclonal anti-DNA-receptor IgG, then fluorescein-conjugated goat anti-mouse IgG. Top: fluorescence; bottom: bright field (half-magnification).

12. ***tat** Protein Binding to RNA*

The plasmid pSP6HIV+1 (Muesing, et al., 1987), which encodes the first 232 nt of all HIV-1 mRNAs, was obtained from Dr. Daniel Capon, used to transform competent *E. coli* DH5$\alpha$ cells. Supercoiled plasmid, isolated from a 1 L culture, was restricted and satisfactorily transcribed in vitro (Fig. 30).

Electrophoresis of mixtures of *E. coli* initiation factor 3 protein with the 3'-terminal 49 nt fragment of *E. coli* 16S rRNA on agarose, according to Lowary and Uhlenbeck (1987), demonstrated positive results (Fig. 31) in an analogous system which we have studied extensively (Wickstrom and Laing, 1988).

When *tat* protein was incubated with the pSP6HIV+1 transcript, or with a similar size transcript from VSV matrix gene, or three similar size *E. coli* transcripts, strong binding of multiple copies of *tat* protein to all sequences was observed (Fig. 32). This result agrees with Frankel, et al. (1988), but seems paradoxical in view of the strong dependence of *tat* activity on the integrity of the loop sequence in the TAR region (Feng and Holland, 1988). Perhaps the TAR loop forms a tertiary interaction with a proximal sequence in the first exon, which is destabilized by nonspecific *tat* binding. If *tat* is just a general single stranded RNA binding protein, then designing a *tat*-specific inhibitor may be more difficult than if *tat* is found to bind to a specific sequence.

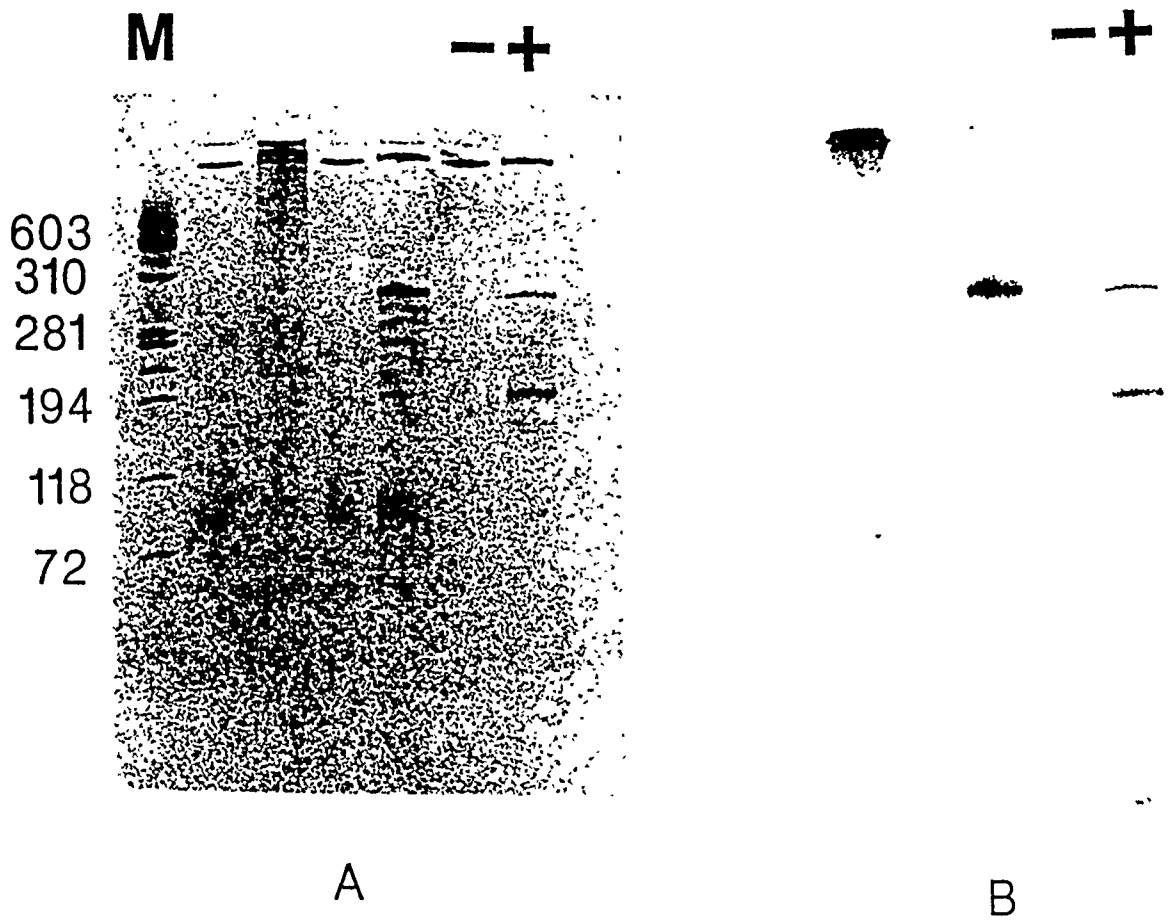


Fig. 30. Transcription of HIV TAR sequence. pSP6HIV+1 (Muesing, et al., 1987) was restricted with Xho I and transcribed with SP6 RNA polymerase, then analyzed by denaturing electrophoresis on 4% polyacrylamide and fluorography of gel stained with ethidium bromide (A), then autoradiography (B). Lane M: ΦX174 DNA cut with Hae III; fragment sizes are shown in bp. Lane -: restricted plasmid alone; lane +: with SP6 polymerase added.

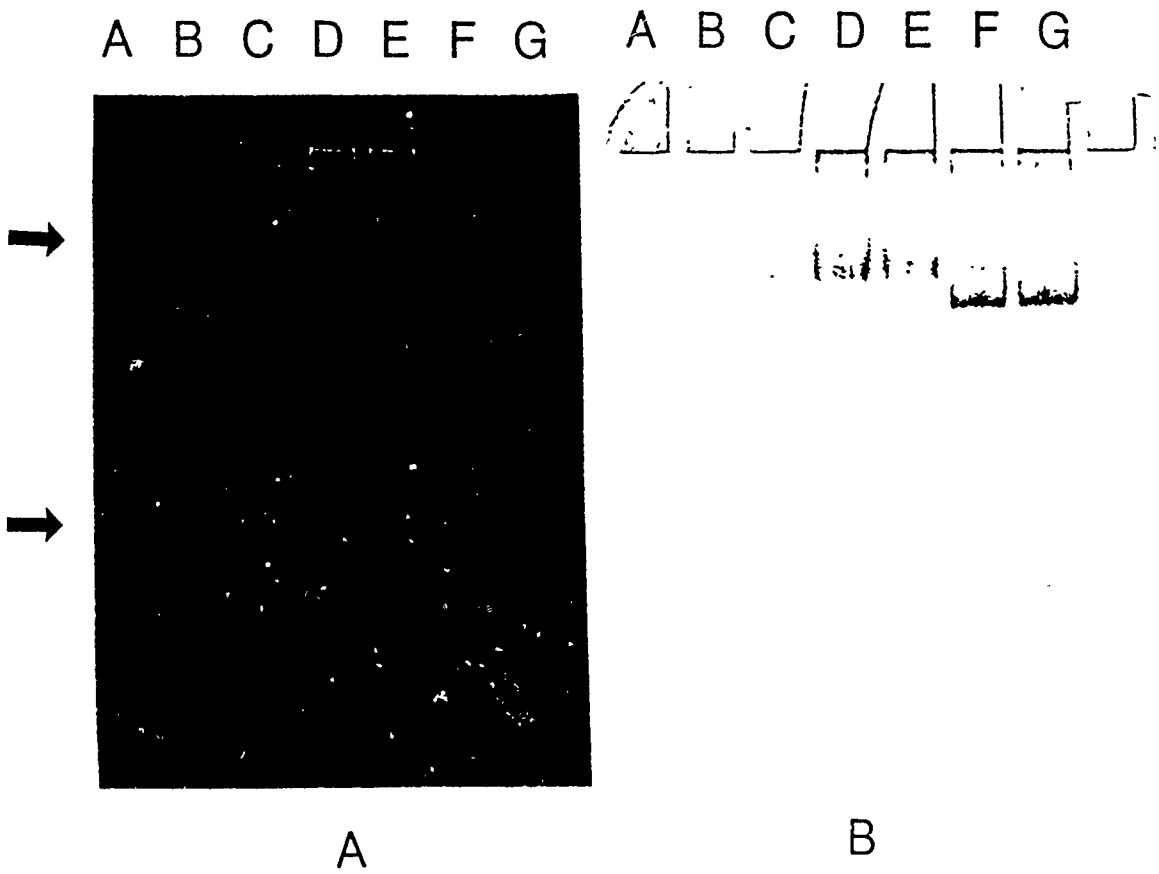


Fig. 31. Gel mobility retardation of RNA by IF3 protein. *E. coli* ribosomes were treated with cloacin DF13 to release the 3'-terminal 49 nt fragment of 16S rRNA. IF3 alone, 10 $\mu$g, was run in lane A, and bovine serum albumin alone, 8 $\mu$g, in lane G. The purified RNA, 1 $\mu$g, was mixed with nothing (B), 1 $\mu$g IF3 (C), 6 $\mu$g IF3 (D), 10 $\mu$g IF3 (E), and 8 $\mu$g bovine serum albumin (F), in a physiological salt buffer, then electrophoresed on 10% polyacrylamide in 50 mM Tris-$H_3BO_3$, pH 8.3, with 1 mM EDTA. The gel was then stained with ethidium bromide, A, then Coomassie blue, B to visualize RNA and protein, respectively. Upper arrow indicates IF3-RNA complex; lower arrow indicates RNA alone.


Fig. 32. Gel mobility retardation of RNA by *tat* protein. Uniformly $^{35}$S-labelled transcripts were incubated without (lanes A, C, E, G, I) or with (lanes B, D, F, H, J) 6 $\mu$M *tat* protein in 50 mM Tris-$H_3BO_3$, 2 mM $Mg(OAc)_2$, 1 mM EDTA, pH 8.3, for 5 min. at 37°, then 30 min. at 0°. Reaction mixtures were then electrophoresed on 6% polyacrylamide in 50 mM Tris-$H_3BO_3$, pH 8.3, 1 mM EDTA, at 4°, and the gel was fluorographed. Transcripts studied included the 213 nt leader of *E. coli infC* (A, B), the 243 nt leader of *E. coli infC* $\alpha$ operon IF3 (C, D), the 3' 295 nt of the VSV M protein gene (E, F), the 232 nt leader of HIV-1 (E, F), and the 212 nt portion of *E. coli* 16S rRNA from nt 679-891 (G, H).

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## I. PUBLICATIONS

1. *Published Papers:*

a. Bacon, T. A., Morvan, F., Rayner, B., Imbach, J.-L., and Wickstrom, E. (1988) "α-Oligodeoxynucleotide Stability in Serum, Subcellular Extracts and Culture Media", *Journal of Biochemical and Biophysical Methods* **16**, 311-318.

b. Lise, L. D., Jolivet, M., Audibert, F., Fernandez, A., Wickstrom, E., Chedid, L., and Schlessinger, D. H. (1989) "Role of the Proline Residues on the Immunogenic Properties of a *P. falciparum* Circumsporozoite Peptide Linked to a Carrier Protein", *Peptide Research* **2**, 114-119.

c. Wickstrom, E. L., Bacon, T. A., Gonzalez, A., Lyman, G. H., and Wickstrom, E. (1989), "Anti-c-*myc* DNA Oligomers Increase Differentiation and Decrease Colony Formation by HL-60 Cells", *In Vitro Cellular and Developmental Biology* **25**, 297-302.

2. *Submitted Papers:*

a. Bacon, T. A., Gonzalez, A., Mahovich, K., and Wickstrom, E. (1989), "Daily Addition of an Anti-c-*myc* DNA Oligomer Induces Granulocytic Differentiation of Human Promyelocytic Leukemia HL-60 Cells in Both Serum-containing and Serum-free Media", *Oncogene Research*, under revision.

b. Bacon, T. A., and Wickstrom, E. (1989) "Walking along human c-*myc* mRNA with antisense oligodeoxynucleotides: maximum efficacy at the 5' cap and initiation codon regions", submitted for publication.

3. *Papers in Preparation:*

a. Zou, A.-Q., Hudson, D., Tsou, D., Cook, R. M., Teplow, D., Wong, H., Jonak, J., and Wickstrom, E. (1989) "Solid Phase Synthesis of Human Immunodeficiency Virus *tat* Protein: Purification and Functional Characterization", manuscript in preparation.

b. Bacheler, L., Looney, D., Wong-Staal, F., and Wickstrom, E. (1989) "Antisense Inhibition of HIV Gene Expression at TAR Targets", manuscript in preparation.

c. Lebedev, A. V., Wenzinger, G., and Wickstrom, E. (1989) "Coupling of 5'-Dimethoxytrityl Deoxynucleoside Methylphonamidites to 3'-Acetyl Deoxynucleosides Without Tetrazole", manuscript in preparation.

4. *Published Abstracts:*

a. Wickstrom, E., Bacon, T. A., Gonzalez, A., and Mahovich, K. (1988) "Antisense DNA Inhibitors of Gene Expression", American Chemical Society, Florida Division, 41st Annual Meeting, Tampa.

b. Israel, J., Norton, D. R., Wenzinger, G. R., and Wickstrom, E. (1988) "Synthesis and Separation of Diastereomers of 5'-O-Dimethoxytrityl Thymidine

Methylphosphonyl Thymidine 3'-O-Acetate", American Chemical Society, Florida Division, 41st Annual Meeting, Tampa.

c. Bacon, T. A., Gonzalez, A. G., Mahovich, K., and Wickstrom, E. (1988) "Down-regulation of Oncogenes and Viral Genes in Transformed Human Cells by Antisense Oligodeoxynucleotides", International Workshop on Therapeutic and Diagnostic Applications of Oligonucleotide Derivatives, Novosibirsk, USSR.

d. Cook, R. M., Hudson, D., Zou, A.-Q., Teplow, D. B., Wong, H., Tsou, D., and Wickstrom, E. (1988) "FMOC Mediated Solid Phase Assembly of HIV *tat*-III Protein", 20th European Peptide Symposium, Tübingen, West Germany.

5. *Invited Seminars:*

a. "Exogenous Antisense DNA Probes of Gene Expression", Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey, 1987.

b. "Antisense Oligodeoxynucleotide Inhibition of Viral and Oncogene Expression", Molecular Therapeutics, Inc., West Haven, Connecticut, 1987.

c. "Exogenous Antisense DNA Probes of Gene Expression", Department of Biochemistry, University of South Florida School of Medicine, Tampa, Florida, 1987.

d. "Potential DNA Drugs for Treatment of Cancer and AIDS", Department of Natural Sciences, Eckerd College, St. Petersburg, Florida, 1987.

e. "Antisense DNA Therapeutics for Hybrid Arrest of Oncogenes and Viral Genes", Biosearch, Inc., San Rafael, California, 1987.

f. "Antisense DNA Therapeutics for Hybrid Arrest of Oncogenes and Human Immunodeficiency Virus Genes", Eastman Pharmaceuticals, Inc., Great Valley, Pennsylvania, 1987.

g. "Use of Antisense DNA Oligomers as Inhibitors of Gene Expression in Cells", Department of Virology, Centers for Disease Control, Atlanta, Georgia, 1988.

h. "Antisense Oligonucleotides as Possible Therapeutic Agents", Department of Molecular Pharmacology, Smith, Kline, and French Laboratories, King of Prussia, Pennsylvania, 1988

i. "Prospects for Antisense DNA Therapeutics", Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, 1988

j. "Antisense Oligodeoxynucleotide Inhibition of HIV Gene Expression", Workshop on Prophylaxis and Therapy of HIV Infections, Walter Reed Army Institute of Research, Washington, District of Columbia, 1988

k. "Efficacy of Antisense Oligodeoxynucleotides Against Oncogene and Viral Gene Expression", Department of Molecular Genetics, Hoffmann-La Roche, Inc., Nutley, New Jersey, 1988

l. "Down-regulation of Human c-*myc* Oncogene and HIV *tat* Gene Expression by Antisense Oligodeoxynucleotides", Public Health Research Institute, New York, New York, 1989.

m. "Antisense DNA Therapeutics", All Children's Hospital, Saint Petersburg, Florida, 1989.

## J. PERSONNEL

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|---|---|
| 1. Dr. Eric Wickstrom | Associate Professor |
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| 3. Dr. An-qing Zou | Research Associate |
| 4. Devon Norton | Research Assistant |
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Graduate Degrees: None

# APPENDIX

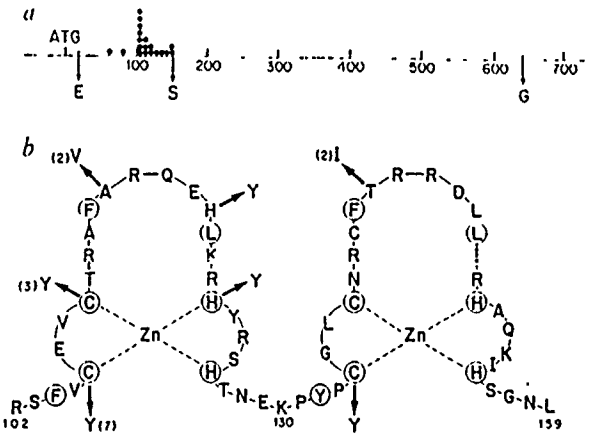


Fig. 3 Positions of mutations in the proposed ADR1 finger structure. *a*, Region of the *ADR1* coding sequence which was mutagenized with hydroxylamine. Mutations (small filled circles) map to a limited region of the *ADR1* coding sequence. Although the DNA corresponding to this whole region was not sequenced, plasmid reconstruction experiments indicated that these mutations fully account for the Adr1⁻ phenotype. The numbers represent ADR1 amino acids. Restriction sites: E, *Eco*RI; S, *Sph*I and G, *Bgl*II. *b*, Amino-acid sequence of the proposed ADR1 finger structure and the positions of *adr1* finger mutations. This structure lies between amino acids 102 and 159 in the ADR1 polypeptide and is drawn according to the model proposed by Miller *et al.*[2]. Invariant cysteine, histidine and hydrophobic residues are circled. Arrows, positions of the ADR1 amino-acid changes in Table 1. The number of independently isolated mutations at each position is given in parentheses.

contiguous repeat units to bind DNA. A number of DNA-binding or transcriptional activation proteins contain only one cysteine-rich repeat unit which resembles that of the finger proteins[6,10-12]. We predict that these proteins will be found to form a different DNA-binding structure than the finger proteins.

Mutations were identified that affected four different amino-acid positions (Cys 106, Cys 109, His 122 and Cys 134) proposed to be zinc ligands (Fig. 3*b*). Replacement of these invariant cysteine or histidine residues with a tyrosine led to an *adr1* null phenotype, consistent with their proposed function in forming a finger structure (Table 1).

Miller *et al.*[2] have proposed that the fingertips in the finger structure make direct contacts with the DNA based on the large number of DNA binding residues found in this region of the TFIIIA fingers. Only one of the mutations in the ADR1 fingertips (His 118 → Tyr) led to an *adr1* null phenotype. His 118 may make a specific contact with DNA or may act in transcriptional activation. The other fingertip mutations, Ala 114 → Val and Thr 142 → Ile, have an unexpected phenotype. They have a more severe effect on *ADH2* expression during growth on glucose containing media than on media containing a non fermentable carbon source. Ala 114 and Thr 142 may also be involved in DNA binding or transcriptional activation but may be less important than His 118.

Analysis of transcription factor TFIIIA led to a proposed model for a DNA binding domain different from the helix-turn-helix motif[2]. Both structural and genetic studies demonstrated the importance of the helix turn helix structure for DNA binding[1]. Although physical evidence supporting the existence of the finger structure is lacking, our genetic data are consistent with the model and strongly support the proposed role of zinc ligands in forming a DNA binding finger domain.

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# A c-*myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from $G_0$ to $G_1$

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**Initiation of T-lymphocyte proliferation by mitogen or antigen involves a cascade of gene activation events. Thus, by the time mitogen-activated T cells have reached the $G_1$/S interface, many genes that are transcriptionally silent in $G_0$, like the c-*myc*, IL-2, IL-2 receptor (IL-2R) and transferrin receptor (TfR) genes, have been transcriptionally activated[1-4]. To understand the role of the individual genes in the activation process, one must be able to interfere specifically with the expression or function of each particular gene product. In this way, by blocking the IL-2R with an antibody, it has been demonstrated that IL-2/IL-2R interaction is required to induce TfR expression in activated T cells[3]. When the function or expression of intracellular proteins is to be blocked, however, the need to introduce antibodies into the cytoplasm of viable cells, although possible[5-7], is a limiting factor. We have taken another approach, namely the exogenous addition to bulk cell cultures of small antisense oligomers. Sequence-specific antisense oligodeoxyribonucleotides have been reported to inhibit intracellular viral replication without interfering with cellular protein synthesis[8,9]. Similarly, rabbit globin mRNA translation in a cell-free system and in rabbit reticulocytes has been inhibited by oligomers complementary to the globin mRNA initiation codon region[10]. Recently, a pentadecadeoxyribonucleotide complementary to the initiation codon and four downstream codons of human c-*myc* mRNA was reported to inhibit the proliferation of the human leukaemic cell line HL-60 specifically[11]. We report here that the same c-*myc* complementary oligonucleotide inhibits mitogen-induced c-*myc* protein expression in human T lymphocytes and prevents S phase entry. Interestingly, c-*myc* antisense treatment did not inhibit $G_0$ to $G_1$ traversal as assessed by morphologic blast transformation, transcriptional activation of the IL-2R and TfR genes, or induction of $^3$H-uridine incorporation.**

The specific role(s) of the c-*myc* gene in lymphocyte mitogenesis is unknown. Initiation of c-*myc* transcription occurs

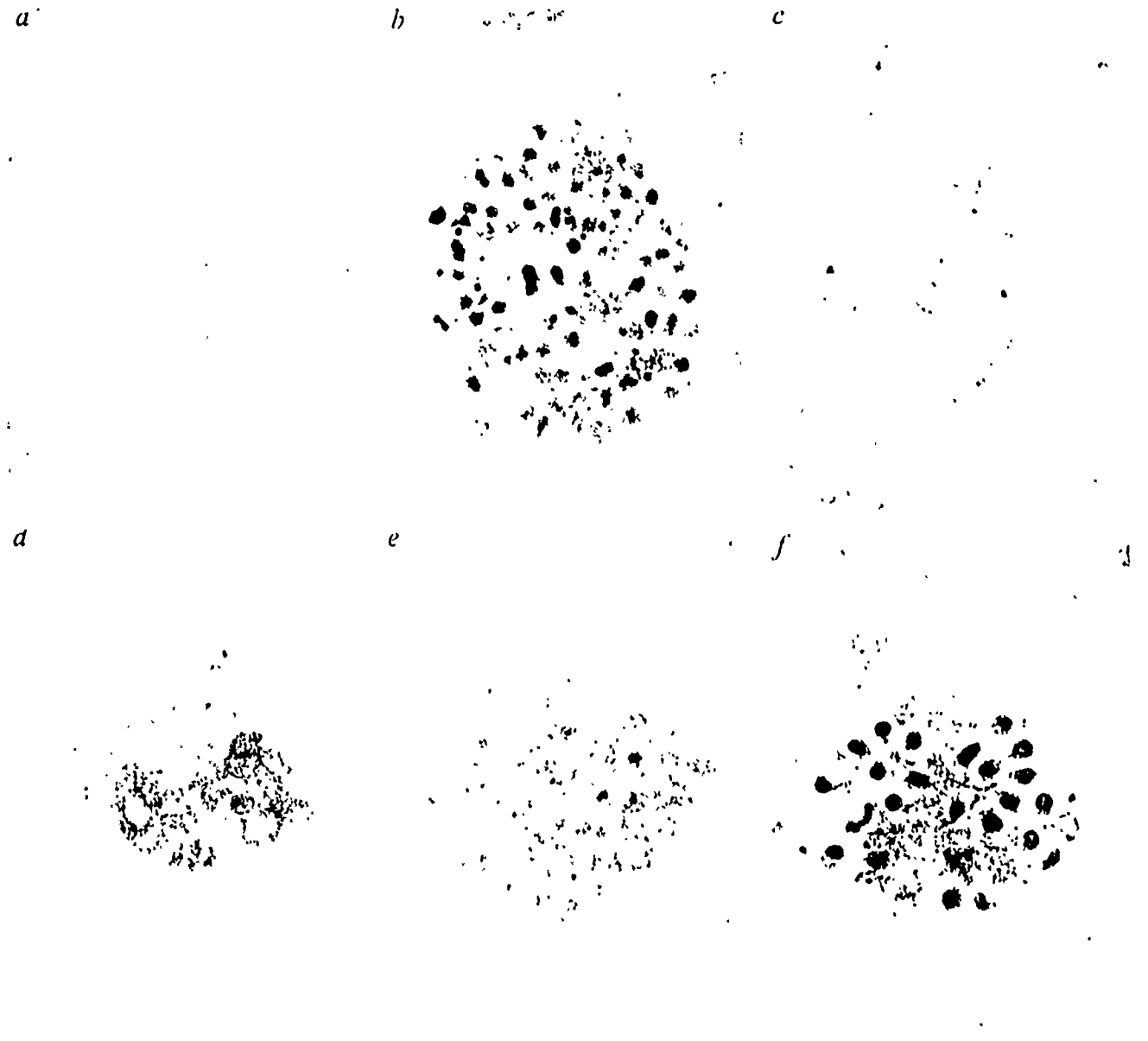


Fig. 1 Inhibition of PHA-stimulated c-*myc* protein expression in peripheral blood lymphocytes by an antisense oligomer to c *myc* mRNA *a*, Unstimulated lymphocytes stained with anti-c-*myc* antibody, *b*, PHA-stimulated cells stained with anti-c-*myc* antibody, *c*, PHA stimulated cells stained with anti-c-*myc* antibody which had been preincubated with a twofold excess of recombinant c-*myc* protein, *d*, PHA stimulated cells stained with irrelevant mouse $IgG_1$, *e*, c-*myc* antisense-pretreated, PHA-stimulated cells stained with anti c-*myc* antibody, *f*, VSV M protein antisense-pretreated, PHA-stimulated cells stained with anti-c-*myc* antibody.

Methods: Peripheral blood mononuclear cells were isolated from 20 ml venous blood of a normal donor by density centrifugation Upon isolation, the cells were comprised of 73% T lymphocytes, 10% B lymphocytes and 17% monocytes by flow cytometric analysis[3] After 10 h of culture, non-adherent cells consisted of 85% T lymphocytes, 10% B lymphocytes and 5% monocytes Cell viability remained greater than 90% under all conditions (as determined by trypan blue exclusion). Cells were cultured (37 °C, 5% $CO_2$, humidified atmosphere) at a concentration of $1 \times 10^6$ ml$^{-1}$ ($10^5$ per well) in a 96-well U-bottom microtitre plate (Costar) in RPMI 1640 containing 10% fetal bovine serum and antibiotics (BRL). Some wells were pretreated with 30 μM antisense oligomer to either human c *myc* mRNA or VSV M protein mRNA Oligomer sequences were complementary to the first 5 codons (starting from the initiation codon) of human c *myc* mRNA[11] and nucleotides 17–31 of VSV M protein mRNA[18,21]. Oligomer synthesis was performed as described elsewhere[24] and oligomers were purified by reverse phase high-pressure liquid chromatography on a $C_{18}$ column eluted with a 0–25% acetonitrile gradient in 50 mM triethyl ammonium acetate (*p*H 6.0) After a 4-h incubation with or without oligomers, PHA (2 μg ml$^{-1}$) was added to all wells except the unstimulated controls Six hours later, cells were removed and centrifuged onto microscope slides with a cytocentrifuge (Shandon, Inc.). After air drying for ~30 min, the slides were fixed for 30 min in freshly prepared paraformaldehyde (1% in Tris-buffered saline). After 3 washes in Tris buffered saline (containing 1% normal goat serum), slides were stained with one of the following. 1, murine monoclonal anti human c-*myc* antibody[19] (1:500 dilution of ascites), 2, an irrelevant mouse ascites preparation of the same immunoglobulin class and concentration, 3, murine anti c *myc* antibody which had been preincubated with a twofold excess of purified recombinant human c *myc* protein[21]. The staining procedure was that of Hsu *et al.*[25], and was performed immediately after fixation as delayed staining resulted in a loss of nuclear localization and appearance of cytoplasmic anti-c-*myc* reactivity.

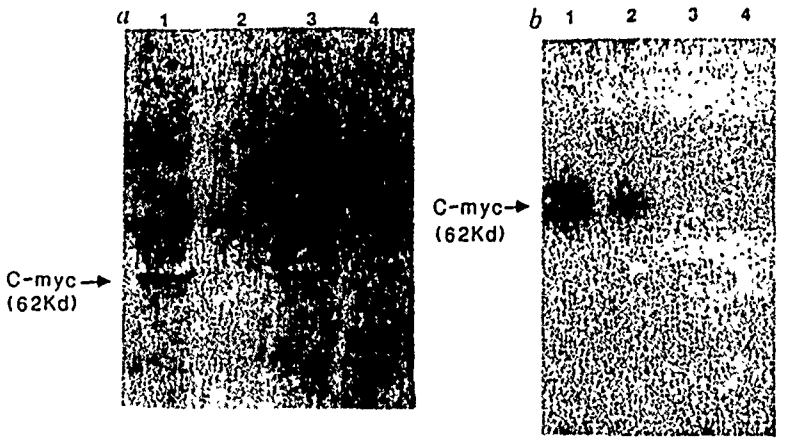


Fig. 2. Inhibition of c-*myc* protein accumulation following PHA-stimulation of peripheral blood lymphocytes by an antisense oligomer of c-*myc*. *a*, Western blot analysis. Lane 1, PHA; lane 2, unstimulated; lane 3, *myc* nonsense pretreated/PHA treated; lane 4, *myc* antisense pretreated/PHA treated. *b*, Immunoprecipitation of metabolically labelled cell lysates. Lane 1, PHA; lane 2, *myc* sense pretreated/PHA treated; lane 3, *myc* antisense pretreated/PHA treated; lane 4, unstimulated.
Methods. *a*, Following treatment conditions as described in Fig. 1, $5\times10^6$ cells were washed twice in cold PBS, solubilized in sample loading buffer (containing 2-mercaptoethanol), electrophoresed on an 8% polyacrylamide gel, transferred to nitrocellulose and reacted with a murine monoclonal anti-*myc* antibody (3C7) as described by Evan *et al.*[19]. The goat anti-mouse second antibody nonspecifically reacted with another band larger than c-*myc*, which could be reduced in intensity by extensive washing of the membrane. This other band does not appear if metabolically labelled samples are immunoprecipitated. *b*, Cells were labelled with 1 mCi each of $^{35}$S-methionine and $^{35}$S-cystein during the time the treatments were administered as in Fig. 1 (10 h). After treatment, $5\times10^6$ cells in each group were lysed in 0.01 M Tris-Cl (*p*H 7.5), 0.144 M NaCl, 0.5% NP-40, 0.5% SDS, 0.1% Trasylol, 1 mM phenylmethylsulphonyl fluoride and 10 mM iodoacetamide. After 30 min on ice (with vortexing every 10 min) the lysates were centrifuged at 10,000*g* for 10 min. The lysates were then adjusted to 1 ml and incubated for 1 h with 0.1 ml of protein A-coated Sepharose beads which had been pretreated with normal mouse serum. After centrifugation, the lysates were incubated overnight with protein-A coated Sepharose beads to which excess 3C7 antibody had been adsorbed. After 5 washes in lysis buffer to which 0.5% deoxycholate had been added, the bead pellets were resuspended in 4× sample loading buffer (containing 2-mercaptoethanol), boiled, electrophoresed and analysed as described by Hann *et al.*[20].

within a few hours of mitogen addition to T lymphocytes and has been suggested to be important in the $G_0$ to $G_1$ transition[12,13]. Interestingly, antibodies directed against the c-*myc* protein inhibit DNA polymerase activity in nuclei isolated from human cells[14]. But accumulation of c-*myc* transcripts is not sufficient to support T-lymphocyte proliferation[15-17]. Whether c-*myc* induction is required for proliferation is not known. We have addressed this question by attempting to inhibit c-*myc* protein expression with antisense oligomers. Freshly isolated peripheral blood lymphocytes, considered to be $G_0$ cells, were used in these experiments. An oligodeoxyribonucleotide complementary to the first five codons of human c-*myc* messenger RNA (5'-dAACGTTGAGGGGCAT-3'), which were predicted to form a large hairpin loop[11], was added to cells at a final concentration of 30 μM 4 hours before addition of the mitogenic lectin, phytohaemagglutinin (PHA-P, Sigma), As a control, the same concentration of a pentadecadeoxyribonucleotide complementary to nucleotides 17-31 of vesicular stomatitis virus (VSV) M protein mRNA was used (5'-dTTGGGATAACACTTA-3'; ref. 18). In addition, oligomers termed 'sense' and 'nonsense' were studied. The c-*myc* sense oligomer was complementary to the antisense construct, while the nonsense oligomer contained the same bases as the antisense construct, but these were randomly scrambled (5'-dCTGAAGTGGCATGAG-3'). Intranuclear c-*myc* protein was detected in cytospin preparations ($10^5$ cells per slide) using a murine monoclonal anti-human c-*myc* antibody (provided by Dr G. Evan[19]). C-*myc* protein was also quantified by Western blot analysis as described by Evan *et al.*[19], and by immunoprecipitation of $^{35}$S-cysteine and $^{35}$S-methionine metabolically-labelled cell lysates as described by Hann *et al.*[20]. Resting ($G_0$) T cells possessed no detectable c-*myc* protein reactivity (Fig 1*a*; Fig. 2*a*, lane 2). However, within 6 h of PHA addition, 30-40% of the cells demonstrated strong nuclear reactivity with the c-*myc* antibody (Fig. 1*b*; Fig. 2*a*, lane 1). Prior incubation of the antibody with recombinant c-*myc* protein[21] completely blocked the staining (Fig 1*c*). Staining with a non-reactive mouse ascites of the same isotype and the same immunoglobulin concentration also produced no reactivity (Fig. 1*d*). Preincubation of the cells for 4 h with 30 μM c-*myc* antisense, markedly reduced the number (to 10%) and intensity of c-*myc* protein positive cells 6 h after PHA addition (Fig. 1*e*; Fig. 2*a*, lane 4). Antisense concentrations lower than 30 μM were less inhibitory, although doses as low as 15 μM produced some diminuation in c-*myc* expression (data not shown). Pretreatment with c-*myc* antisense markedly inhibited the synthesis of c-*myc* protein as detected by immunoprecipitation of metabolically labelled material (Fig. 2*b*). Preincubation with VSV M protein antisense, c-*myc* sense, or c-*myc* nonsense oligomers had little inhibitory effect on c-*myc* expression (Fig. 1*f*; Fig. 2*a*, lane 3; Fig. 2*b*, lane 2).

At 20 h after mitogen stimulation, the c-*myc* protein level of control lymphocytes was lower than at 6 h, but it was still inhibited in the c-*myc* antisense pretreated cells (data not shown). Thus, consistent with the findings of Persson *et al.*[4], c-*myc* protein levels follow a time course similar to that previously reported for c-*myc* gene transcription[1,12], and one addition of antisense oligomer is sufficient to inhibit appearance of the c-*myc* protein substantially throughout this time course. But if PHA is added 24 or 48 h after oligomer pretreatment, c-*myc* protein can be induced to the same degree as in the PHA control samples (data not shown). These results, taken together with viability studies (by trypan blue exclusion) made at 72 h after PHA addition, suggest that oligomer addition itself is not toxic and that the oligomer is sufficiently degraded within about 24 h to be ineffective.

Using c-*myc* antisense, we then examined the effects of inhibition of c-*myc* protein expression on PHA-stimulated cell proliferation. Initial attempts to study proliferative capacity using $^3$H-thymidine proved impossible as both oligomers reduced $^3$H-thymidine incorporation into cellular DNA by >80% (cells were pulsed at +72 h for 4 h with 1 μCi $^3$H-thymidine). Synthetic oligomers have previously been reported to reduce $^3$H-thymidine incorporation into cellular DNA nonspecifically[8]. Eighteen hours after addition of d-$(Tp)_8$[$^3$H]T, a tritiated dT homo-octamer, to mammalian cells, only 70% of the label remained associated with the oligomer, whereas the other 30% was found associated with thymidine triphosphate and cellular DNA, indicating the occurrence of intracellular oligomer degradation with subsequent reuse of the thymine base[22]. When a d-$(Ap)_8$A (dA homo-octamer) analogue was tested, it had no effect on $^3$H-thymidine incorporation assays[22]. As the oligomer complementary to c-*myc* contains three T's and the oligomer complementary to VSV M protein contains five T's, these reagents could significantly increase the intracellular thymidine pool at +72 h, thereby greatly diluting the specific activity of this pool after pulse-labelling with 1 μCi exogenous thymidine. To cir-

Table 1 Effect of an antisense oligodeoxyribonucleotide complementary to c-*myc* mRNA on mitogen-stimulated lymphocyte proliferation

| | $G_0/G_1$ (%) | S (%) | $G_2$/M (%) | Mitotic index (%) |
|---|---|---|---|---|
| Untreated cells | 78 (78, 78) | 8 (7, 9) | 14 (13, 15) | <1 |
| PHA-stimulated cells | 53 (52, 54) | 24 (20, 27) | 24 (21, 26) | 5 |
| PHA-stimulated cells Pretreated with antisense to VSV M protein mRNA | 50 (50, 50) | 30 (30, 30) | 20 (20, 20) | 4 |
| PHA-stimulated cells pretreated with antisense to c-*myc* mRNA | 78 (73, 82) | 7 (3, 10) | 16 (15, 17) | <1 |

Freshly isolated normal peripheral blood lymphocytes were cultured as described in the legend to Fig. 1. Some wells were pretreated for 4 h with 30 μM oligomer complementary to either c-*myc* or VSV M protein mRNA. All wells except the unstimulated controls then received 2 μg ml$^{-1}$ PHA. Three days later, cells were removed and DNA cell-cycle phase analysis was performed according to the method of Braylan *et al.*[26]. Essentially, cells were washed in phosphate-buffered saline, fixed for 1 h in 50% ethanol at 4 °C, incubated with 500 units ml$^{-1}$ RNase A (Worthington; boiled to destroy contaminating DNase activity) for 30 min at 37 °C, and incubated for 30 min at room temperature with propidium iodide (25 μg ml$^{-1}$, Calbiochem). Samples were analysed on a FACS II flow cytometer (Becton-Dickinson FACS Systems) using the 488-nm line of an argon laser for excitation. Quantification of cell-cycle phase distributions was performed on an integrated PDP 11/34 computer (DEC) using software developed by the Division of Computer Research and Training, NIH[27]. We have routinely observed that 75–85% of freshly isolated peripheral blood lymphocytes are in $G_0/G_1$, whereas 45–55% of continuously proliferating cells are in $G_0/G_1$ at any given time. The cell-cycle data are expressed as the mean of two separate experiments with the individual values in parentheses. Mitotic indices were determined on 72-h PHA-stimulated samples with or without antisense oligomers. Cells were centrifuged onto glass slides and stained with Wright's stain. The number of mitoses per 1,000 cells was then determined microscopically. The mitotic index for PHA activated lymphocytes typically averages about 5% (ref. 28).


Fig. 3 PHA-stimulated blastogenesis after c-*myc* antisense pretreatment. Peripheral blood lymphocytes were isolated and cultured as described in the legend to Fig. 1 and Table 1. Three days after PHA treatment, cells were centrifuged onto glass slides and stained with Wright's stain to reveal basic cellular morphology. Pretreatment with antisense oligomer to either c-*myc* (*c*) or VSV M protein (*d*) did not prevent PHA-stimulated blastogenesis (*b*) when compared to unstimulated controls cultured for 3 days (*a*).

cumvent this difficulty, we assessed proliferation using two independent techniques—DNA cell-cycle analysis and determination of mitotic index.

DNA histograms were generated at 72 h after PHA addition (Table 1). Cells were fixed, reacted with the DNA intercalating dye propidium iodide and analysed by flow cytometry. Of the untreated cells, 78% remained in $G_0/G_1$ at this time, whereas only 53% of the stimulated cells did so. Although pretreatment with VSV M protein antisense had no effect on the proliferation of PHA-treated cells (50% in $G_0/G_1$), pretreatment with c-*myc* antisense resulted in 78% of the cells remaining in $G_0/G_1$ 72 hours after mitogen addition. These results were confirmed by quantifying the mitotic indices of cell populations 72 h after PHA stimulation in the presence or absence of antisense oligomers (Table 1). The mitotic index is another measure of a cell population's proliferative capacity because mitoses can be observed only in cells which have attained a tetraploid DNA content before actual cell division. Mitotic indices were determined from Wright's stained-cell preparations similar to those pictured in Fig. 3 and were found to be reduced by more than 80% in c-*myc* antisense pretreated cultures (comparable to the frequency seen in unstimulated cells). VSV M protein antisense pretreatment did not affect the mitotic index. C-*myc* antisense pretreatment thus inhibited PHA-stimulated DNA synthesis, indicating that c-*myc* protein expression is required for this to occur.

We next studied the effects of inhibition of c-*myc* protein expression on $G_0$ to $G_1$ traversal by following the morphological changes induced by PHA in the presence and absence of c-*myc* antisense pretreatment, the expression of the proliferation-associated genes for IL-2R and TfR, which are known to be activated subsequent to c-*myc*[1]; and induction of $^3$H uridine incorporation. Expression of IL-2R and TfR was examined 48 h after PHA addition. Prior exposure to c-*myc* antisense did not block the mitogen stimulated appearance of these receptors on the cell surface (Table 2). As TfR is known to be expressed after Il-2R expression in late $G_1$ (refs 1, 3, 16), our data indicate that even when c-*myc* protein expression is substantially reduced, the cells are able to traverse from $G_0$ to $G_1$ and into late $G_1$. It has been shown previously that IL-2R may be induced in the absence of c-*myc* induction[2], consistent with the fact that IL 2R gene transcriptional activation is not dependent on protein synthesis[1]. Our results suggest that TfR expression is also independent of c-*myc* expression, although we cannot rule out a dependence on the very low levels of c-*myc* protein observed in c-*myc* antisense-treated cells. On the other hand, IL-2R and TfR expression are not sufficient to support DNA synthesis in the absence of normally induced amounts of the c-*myc* protein.

Uridine incorporation was monitored 18–24 and 24–48 h after PHA stimulation (Table 2). C-*myc* antisense pretreatment failed to block PHA-stimulated induction of $^3$H uridine incorporation measured at either time point, supporting our hypothesis that c-*myc* antisense-pretreated cells are capable of entering $G_1$ upon PHA stimulation. In accordance with this observation, the morphological alterations characteristic of lymphocyte

**Table 2** Effects of an antisense oligodeoxyribonucleotide complementary to c-*myc* mRNA on mitogen-stimulated IL-2 receptor expression, transferrin receptor expression and $^3$H-uridine uptake

| | % Positive cells | | $^3$H-uridine |
|---|---|---|---|
| | IL-2 receptor | Transferrin receptor | incorporation (c.p.m.) |
| Unstimulated cells | 8 | 1 | 506 |
| | (8, 8) | (0, 1) | (424-911) |
| PHA-stimulated cells | 40 | 30 | 1091 |
| | (32, 47) | (23, 37) | (898-2726) |
| PHA-stimulated cells pretreated with antisense to c-*myc* mRNA | 33 | 21 | 1454 |
| | (30, 35) | (16, 26) | (953-1470) |
| PHA-stimulated cells pretreated with antisense to VSV M Protein | 37 | 26 | 1197 |
| | (31, 42) | (23, 29) | (1011-1324) |

Peripheral blood lymphocytes were cultured as described in legend to Fig. 1. Two days after PHA treatment (2 μg ml$^{-1}$) cells were analysed for surface expression of IL-2 receptors and transferrin receptors using the monoclonal antibodies anti-Tac (a gift of Dr T. Waldmann) and OKT9 (Ortho Immunology Systems) respectively. Analyses were performed using a FACS II flow cytometer as described elsewhere[3]. Data are expressed as the mean percent positive cells from two experiments with the individual values in parentheses. Uridine incorporation was determined by labelling cells with 1 μCi ml$^{-1}$ $^3$H-uridine (Dupont/NEN, 38.9 Ci mmol$^{-1}$) within 18-24 h of PHA treatment. Cells were harvested by precipitation with trichloroacetic acid and filtration through Whatman GFC filter disks. Acid precipitable radioactivity was determined by liquid scintillation counting. Data are expressed as the median value of three determinations with the range in parentheses. Uridine incorporation was also determined within 24-48 h of PHA addition. Acid-precipitable radioactivity was then increased 7.8-fold above control in the PHA group, 5.6-fold in the c-*myc* antisense group and 4.5-fold in the VSV antisense group.

mitogenesis were not blocked by c-*myc* antisense pretreatment (Fig. 3). The cells underwent a marked increase in size and decrease in nuclear to cytoplasmic ratio following PHA treatment. Furthermore, the metabolic rate of c-*myc* antisense pretreated cells (as measured by *p*H of the culture medium) was as great as that of the PHA controls (data not shown). These results do not support a role for c-*myc* in the $G_0$ to $G_1$ transition process of normal T lymphocytes, unless the greatly diminished level of c-*myc* protein detected in the c-*myc* antisense treated cells is sufficient Rather, the c-*myc* gene product would appear to be critical for S phase entry and/or S phase traversal. This would be consistent with the recently suggested involvement of the c-*myc* protein in DNA synthesis[14].

We have shown that antisense oligonucleotides can be used to inhibit the expression of the c-*myc* protein in mitogen-stimulated lymphocytes, allowing study of the role of c-*myc* gene expression in lymphocyte mitogenesis. Furthermore, the use of sequence specific antisense oligomers may allow study of the functions of other intracellular proteins.

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# Kinetics of flight muscles from insects with different wingbeat frequencies

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**Small insects usually beat their wings at a higher frequency than large insects. For energetic reasons, the wingbeat frequency is close to the natural resonant frequency of the wing movement[1-3]. In the flight muscles of many insects, termed fibrillar muscles, the contractions are myogenic: the muscle is only partially activated by calcium; full activation requires mechanical stretch. Demembranated fibres can be stretch-activated *in vitro*, allowing both tension changes and the ATPase activity of the fibres to be monitored. The tension response to a sudden length change shows a large delayed tension component (Fig. 1), whose rate-constant determines the frequency at which the muscle can deliver maximum power to the wings. We show here that this rate-constant is roughly proportional to wingbeat frequency in the flying insect. We find that the ATPase rate is slower and is not related to the rate-constant of tension production, and show that insects with widely different wingbeat frequencies have muscles with similar mechanical power output. We conclude: (1) that the rate-limiting step in the cross-bridge cycle, which limits the ATPase activity, follows the state in which maximum active tension is produced and does not limit the rate at which tension is generated; (2) that the first tension-generating state is probably an actomyosin.ADP.Pi state; and (3) that a preceding step, probably an actomyosin.ADP.Pi isomerization, which controls the rate-constant for force generation, is faster in smaller insects than in larger ones.**

We recorded the wingbeat frequencies of different insect species either by tape-recording the sound of the flying insect and then replaying the tape through an oscilloscope, or by mounting the insect on a gramophone pickup and recording on an oscilloscope; or by both methods. We were not able to induce flight in the waterbug *Lethocerus colossicus*; the wingbeat frequencies of species of similar size have been measured previously[4]. The ambient temperature was about 25 °C.

We performed mechanical and biochemical experiments on single demembranated muscle fibres that had been dissected from the insect thorax and mounted on a mechanical testing

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# Human promyelocytic leukemia HL-60 cell proliferation and c-*myc* protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-*myc* mRNA

(antisense DNA/hybrid-arrested translation/oncogenes)

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**ABSTRACT** **The human promyelocytic leukemia cell line HL-60 overexpresses the c-*myc* protooncogene. A calculated secondary structure for c-*myc* mRNA placed the initiation codon in a bulge of a weakly base-paired region. Treatment of HL-60 cells with 5′ d(AACGTTGAGGGGCAT) 3′, complementary to the initiation codon and the next four codons of c-*myc* mRNA, inhibited c-*myc* protein expression in a dose-dependent manner. However, treatment of HL-60 cells with 5′ d(TTGGGATAACACTTA) 3′, complementary to nucleotides 17–31 of vesicular stomatitis virus matrix protein mRNA, displayed no such effects. These results agree with analogous studies of normal human T lymphocytes [Heikkila, R., Schwab, G., Wickstrom, E., Loke, S. L., Pluznik, D. H., Watt, R. & Neckers, L. M. (1987) *Nature (London)* 328, 445–449], except that only one-third as much oligomer was needed for a comparable effect. Proliferation of HL-60 cells in culture was inhibited in a sequence-specific, dose-dependent manner by the c-*myc*-complementary oligomer, but neither the oligomer complementary to vesicular stomatitis virus matrix protein mRNA nor 5′ d(CATTTCTTGCTCTCC) 3′, complementary to nucleotides 5399–5413 of human immunodeficiency virus *tat* gene mRNA, inhibited proliferation. It thus appears that antisense oligodeoxynucleotides added to *myc*-transformed cells via culture medium are capable of eliciting sequence-specific, dose-dependent inhibition of c-*myc* protein expression and cell proliferation.**

Normal proliferative genes that are abnormally active in cancerous cells are called protooncogenes (1). Cell transformation may be caused by inappropriate expression or response of protooncogene products during cell proliferation. Amplification, translocation, overexpression, or abnormal regulation of the the protooncogene c-*myc* has been observed in a wide variety of human leukemias and solid tumors (1, 2)

The c-*myc* protooncogene is an evolutionarily conserved gene found in all vertebrates, and blot hybridization of electrophoretically fractionated RNA indicates that c-*myc* mRNA is expressed in most normal dividing cells (1) constitutively throughout the cell cycle (3). The c-*myc* gene codes for a 49-kDa polypeptide and expresses a nuclear protein with an electrophoretic apparent molecular mass of 65 kDa (p65) (4). Immunostaining studies indicate that p65 is located in the same subnuclear compartments as small nuclear ribonucleoproteins (5). The hydropathy profile of p65 shows 68% homology with the 289 amino acid protein encoded by early region 1A of adenovirus type 5 and significant homology with polyoma large tumor antigen (6). Either of the latter nuclear proteins may collaborate with c-*ras* to transform primary cell cultures (7, 8), analogously to c-*myc*.

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Inducing quiescent cells to proliferate leads to a rapid accumulation of c-*myc* mRNA upon entering the cell cycle, followed by a decline prior to the onset of DNA synthesis (9). However, DNA synthesis may be suppressed by antibodies against c-*myc* protein, which suggests that the c-*myc* protein may be required for entry into S phase (10). Furthermore, c-*myc* mRNA levels decline in HL-60 promyelocytic leukemia cells that have been induced to differentiate into granulocytes by exposure to dimethyl sulfoxide ($Me_2SO$) (11), although it is not known whether the reduction in nuclear p65 modulates the differentiation or is simply another manifestation of cellular differentiation. Hence, it is possible that reducing the level of c-*myc* mRNA translation may be sufficient to induce differentiation and reverse transformation.

Antisense oligodeoxynucleotides were first used for hybridization arrest of Rous sarcoma virus (RSV) gene expression in chicken embryo fibroblasts (12), and comparable results were obtained for another retrovirus, human immunodeficiency virus (13). Intercalating derivatives have shown similar activity against some viral genes (14), whereas alkylating derivatives have been found effective against two neoplastic cell lines (15). Nuclease-resistant oligo(deoxynucleoside methylphosphonate) derivatives have proved useful for antisense inhibition of globin, herpes simplex virus 1, and vesicular stomatitis virus (VSV) (16–18).

When the secondary structure of VSV matrix (M)-protein mRNA was calculated, nucleotides (nt) 17–31 were predicted to occur in a helical region, whereas nt 34–48, including the initiation codon, were predicted to occur in a loop. The pentadecamer complementary to nt17–31 of VSV M-protein mRNA appeared to inhibit translation of all VSV messages in rabbit reticulocyte lysate nonspecifically, whereas the pentadecamer complementary to nt 34–48 inhibited M-protein expression specifically (19). In the studies reported here, an antisense oligodeoxynucleotide directed against a predicted hairpin loop containing the initiation codon of human c-*myc* mRNA elicited sequence-specific, dose-dependent inhibition of c-*myc* gene expression and cell proliferation in c-*myc*-transformed cells.

## MATERIALS AND METHODS

**Secondary Structure and Thermodynamic Predictions.** Folding of the 2121-nt c-*myc* mRNA from K562 cells (20) was predicted using the algorithms of Jacobson *et al.* (21), with the nearest-neighbor-dependent free energies of Freier *et al.* (22) at 37°C in 1 M NaCl. Thermodynamic calculations

Abbreviations. p65, 65-kDa c-*myc* protein, VSV, vesicular stomatitis virus; M protein, matrix protein; $Me_2SO$, dimethyl sulfoxide; nt, nucleotide(s).

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of association constants for synthetic antisense oligodeoxynucleotides hybridizing to single-stranded regions of mRNA were based on the same free energies. No correction of the free energies was made for RNA·DNA hybridization, instead of RNA·RNA hybridization, nor was any correction made for topological constraints on RNA loops or bulges.

**Cell Culture.** HL-60 cells (23) were grown in RPMI 1640 medium with 10% or 20% heat-inactivated fetal bovine serum, and Q8/MC29 cells (24) were grown in RPMI 1640/Dulbecco's modified Eagle's medium (1:1) with 5% heat-inactivated newborn calf serum, 1% chicken serum, and 0.1% $Me_2SO$, with $10^5$ units of penicillin and 0.1 g of streptomycin per liter in both media. Cells were incubated at 37°C in 5% $CO_2$ saturated with water vapor and were maintained in logarithmic growth phase; titers of viable cells were determined by counting trypan blue-excluding cells in a hemocytometer.

**Oligodeoxynucleotide Synthesis, Cellular Uptake, and Stability.** Pentadecamers were prepared for antisense hybridization-arrest experiments. This length was selected in order to allow very strong binding to single-stranded regions of mRNA, or adequate binding to regions that already have some secondary structural or tertiary structural limitations. A probe with 15 residues should be just barely sufficient for theoretical uniqueness in the human genome, particularly for those sequences expressed as mRNA. Previous investigations have demonstrated efficacy with antisense oligodeoxynucleotides of 8–26 residues (12–29). Oligodeoxynucleotides were synthesized (25) automatically and purified by reversed-phase liquid chromatography or gel electrophoresis. Their sequences were checked by chemical sequencing (26). For studies of oligodeoxynucleotide uptake and stability in cells, oligodeoxynucleotides were 5'-labeled with 5'-[$\gamma$-[$^{35}$S]thio]ATP (1276 Ci/mmol; 1 Ci = 37 GBq; du Pont/New England Nuclear) by use of bacteriophage T4 polynucleotide kinase and were purified by denaturing gel electrophoresis (27). For each time point, $5 \times 10^5$ cpm of 5'-$^{35}$S-labeled oligodeoxynucleotide was added to $4 \times 10^6$ HL-60 cells in 0.5 ml of RPMI 1640 with 10% heat-inactivated fetal bovine serum. Each sample was incubated at 37°C for 1, 4, 8, or 24 hr and then sedimented 3 min at 15,000 × *g*. The supernatant was removed and saved, and the cell pellet was washed once in 0.5 ml of phosphate-buffered saline (10 mM $Na_2HPO_4$, pH 7.4/150 mM NaCl) and sedimented again. The supernatant was removed and saved, and the cell pellet was lysed in 0.1 ml of Tris-buffered saline (10 mM Tris·HCl, pH 7.4, 150 mM NaCl) with 1% $NaDodSO_4$ and then extracted with 0.1 ml of phenol. The aqueous phase was removed, and the phenol phase was extracted with 0.1 ml of water. Aliquots of the combined aqueous extracts, cell wash, and culture medium supernatant were analyzed by liquid scintillation counting. The percent of oligodeoxynucleotides taken up by the cells was calculated by dividing counts in the combined aqueous phases of the cell-pellet extract by the total of the counts in the cell pellet, cell wash, and culture medium supernatant. To determine oligodeoxynucleotide stability, aliquots of the combined aqueous phases, and of the culture medium supernatant fraction, were lyophilized, redissolved in 30 $\mu$l of 80% deionized formamide including 0.01% xylene cyanole FF and 0.01% bromophenol blue, and then electrophoresed in a denaturing 20% polyacrylamide gel and fluorographed.

**Hybridization Arrest of Translation.** Translation of c-*myc* mRNA into p65 was detected by indirect immunofluorescence and radioimmunoprecipitation. For indirect immunofluorescence, samples of $1.0 \times 10^6$ HL-60 cells were each resuspended in 1 ml of fresh medium and grown for 6 hr in the presence of target and control oligodeoxynucleotides added to the culture medium. Each sample was pelleted, washed, fixed with 1% paraformaldehyde, pre-adsorbed with 1% normal goat serum, incubated with normal rabbit IgG (Sigma), rabbit anti-$\beta$-actin (ICN), or rabbit anti-p65 (28) followed by fluorescein-conjugated goat anti-rabbit IgG (Sigma), washed, and resuspended in 1% paraformaldehyde, as described (29), and then was observed by light and fluorescence microscopy. For radioimmunoprecipitation, samples of $2.0 \times 10^6$ HL-60 cells were each resuspended in 2 ml of fresh medium and grown for 6 hr in the presence of target and control oligodeoxynucleotides added to the culture medium. Each sample was sedimented after 6 hr to remove medium containing oligodeoxynucleotide, washed, and then resuspended in 0.5 ml of cysteine-free RPMI 1640 (GIBCO) supplemented with [$^{35}$S]cysteine 1022 Ci/mmol; du Pont/New England Nuclear) at 250 $\mu$Ci/ml. Each culture was grown for an additional 1.5 hr; then the cells were sedimented and lysed and the lysates were analyzed by immunoprecipitation followed by electrophoresis and fluorography as described (30). Fluorograms were scanned with an LKB Ultroscan laser densitometer.

**Inhibition of Cell Proliferation.** HL-60 cells were diluted to $10^5$ per ml in 1.0-ml aliquots of growth medium supplemented with antisense oligodeoxynucleotides. Each sample was dispensed in 0.30-ml aliquots into 3 wells of a 96-well microtiter plate. After 5 days of growth, the cells in each well were resuspended in 0.3 ml of phosphate-buffered saline, and 0.1-ml aliquots were removed for staining with 0.1 ml of 0.4% trypan blue and counting. Treated and untreated cells showed 98–100% viability after 5 days of growth, and untreated cells typically showed a 10-fold higher titer, about $10^6$ cells per ml. Q8/MC29 cells were treated similarly, multiplying 10-fold in only 3 days. At this point, the culture medium was aspirated off, and the cells in each well were trypsinized and resuspended in 0.30-ml of phosphate-buffered saline prior to counting as above. Cell counts were converted to percent inhibition by $100 \times (N_n - N)/(N_n - N_0)$, where $N_0$ is the normal titer at the beginning of the experiment, $N_n$ is the titer for untreated cells after $n$ days of growth, and $N$ is the titer for treated cells after $n$ days. Error bars on points represent one standard deviation.

## RESULTS

**Prediction of Antisense Targets and Thermodynamics.** Calculation of a predicted secondary structure for the entire 2121-nt human c-*myc* mRNA from K562 human erythroleukemia cells (20) placed the initiation codon at the beginning of a large bulge loop in a weakly base paired region (result not shown), suggesting that these codons might be readily available for hybridization arrest by an antisense oligodeoxynucleotide. A preliminary calculation of the secondary structure of 400 nt of the same mRNA, centered on the initiation codon, similarly placed the initiation codon and eight following codons in a bulge loop, though differences were apparent in neighboring secondary structure domains due to long-range interactions in the messenger (31).

From the predicted structure, the initiation codon and the next four codons, nt 559–573 of human c-*myc* mRNA (20), were selected as a single stranded target, and the complementary pentadecadeoxynucleotide, 5' d(AACGTTGAGGGGCAT) 3', hereafter called the anti c-*myc* oligomer, was synthesized. In order to control for sequence specific effects, two other pentadecadecamers were prepared, the anti-VSV oligomer, 5' d(TTGGGATAACACTTA) 3', complementary to nt 17–31 of VSV M-protein mRNA (32), which nonspecifically inhibited translation of VSV mRNAs (19) but differed from the anti-c-*myc* sequence in 13 out of 15 residues, and the anti-*tat* oligomer, 5' d(CATTCTTGCTCTCC) 3', complementary to nt 5399–5413 of the human immunodeficiency virus *tat* gene (33), differing in 12 out of 15 residues.

Thermodynamic calculations for the association of 5′ d(AACGTTGAGGGGCAT) 3′ with its complement on c-*myc* mRNA yielded an association constant of $3.2 \times 10^{16}$. This approximate result suggests that stoichiometric inhibition should be possible (i.e., at a ratio of one oligodeoxynucleotide per mRNA) for cells in culture in an ideal case assuming no degradation of oligodeoxynucleotide, efficient cellular uptake of oligodeoxynucleotides, uninhibited diffusion of oligodeoxynucleotides through the cytoplasm, and no competition of oligodeoxynucleotides with initiation-factor proteins or ribosomes.

**Oligodeoxynucleotide Uptake and Stability.** Exogenously introduced oligodeoxynucleotides would not be very effective agents for hybridization arrest, no matter how stable their complexes with mRNA, if they were rapidly hydrolyzed. In previous work, degradation of oligodeoxynucleotides was not seen in rabbit reticulocyte lysate or Dulbecco's modified Eagle's medium with 5% fetal bovine serum over 2 hr at 37°C, but degradation was complete within that time in a HeLa-cell postmitochondrial supernatant and was complete within 15 min in undiluted fetal bovine serum (27). To examine oligodeoxynucleotide uptake by HL-60 cells, aliquots of labeled anti-c-*myc* oligomer or anti-*tat* oligomer were added to HL-60 cells in culture medium and incubated for up to 24 hr. Radioactivity retained by the washed cell pellets was compared with that left in the culture medium (Fig. 1). In RPMI 1640 with 10% heat-inactivated fetal bovine serum, 1–2% of the labeled oligomers were found associated with the cell pellet after 4 hr, and this amount remained about the same up to 24 hr.

Denaturing gel electrophoresis of labeled oligomers remaining in the culture supernatant revealed significant loss of oligodeoxynucleotide within 1 hr, and disappearance was virtually complete by 8 hr (Fig. 2). In the washed cell pellet, however, labeled oligodeoxynucleotides survived intact for up to 24 hr, decreasing to about one-quarter of the original intensity. These observations agree with an earlier report of oligodeoxynucleotide uptake by HeLa cells and chicken embryo fibroblasts in culture (13).

**Inhibition of c-*myc* Protein Expression.** When HL-60 cells in culture were treated with the anti-*myc* or the anti-VSV oligomer and analyzed by indirect immunofluorescence, neither sequence decreased the level of actin, which was detected only in the cytoplasm (Fig. 3 *a* and *d–g*). HL-60 cells treated with the anti-VSV oligomer showed the same level of c-*myc*-encoded p65 protein in the nucleus as untreated cells (Fig 3 *c*, *h*, and *i*). HL-60 cells treated with 6 μM anti-c-*myc* oligomer showed some reduction in the level of nuclear p65 protein, and significant reduction was observed in cells treated with 10 μM anti-c-*myc* oligomer (Fig. 3 *j* and *k*). Radioimmunoprecipitation, however, showed no significant reduction of p65 antigen with 5 μM anti-*myc* oligomer, and about 50% reduction at 10 μM (Fig. 4). The anti-VSV oligomer caused no significant reduction in p65 antigen.

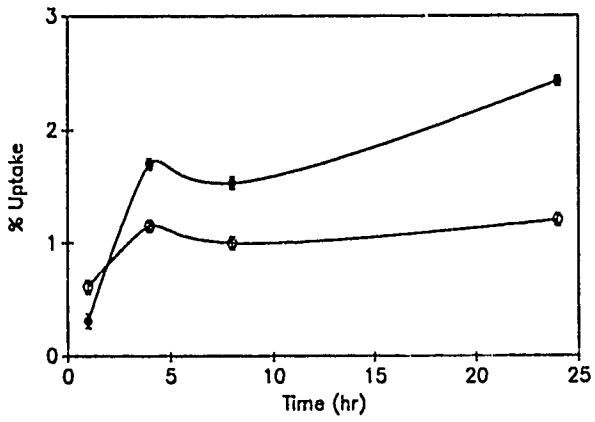


FIG. 1. Uptake of oligodeoxynucleotides by HL-60 cells. Samples of 5′-$^{35}$S-labeled anti-c-*myc* oligomer (●) and anti-*tat* oligomer (○) were incubated with HL-60 cells for 0, 1, 4, 8, and 24 hr. Cells were separated from culture medium by sedimentation, lysed, and extracted with phenol, and oligodeoxynucleotide uptake was determined by liquid scintillation counting of the aqueous phases, as described in *Materials and Methods*.

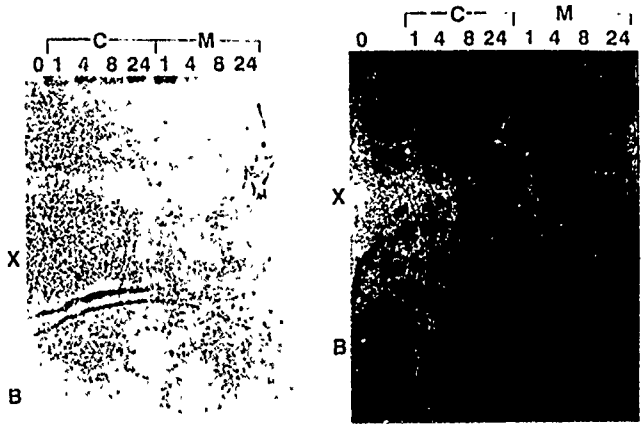


FIG. 2. Stability of oligodeoxynucleotides in HL-60 cells. Samples of 5′-$^{35}$S-labeled anti-c-*myc* oligomer (*Left*) and anti-*tat* oligomer (*Right*) were incubated with HL-60 cells for 0, 1, 4, 8, and 24 hr. Cells were separated from culture medium by sedimentation, lysed, and extracted with phenol, and the aqueous phases were analyzed by denaturing gel electrophoresis, as described in *Materials and Methods*. C, washed cell pellet; M, supernatant medium; numbers, time (hr) of incubation; X and B, mobility of xylene cyanole FF and bromophenol blue, respectively.

**Inhibition of Transformed-Cell Proliferation.** Since c-*myc* p65 antigen appears to be necessary for DNA synthesis (10), it was of interest to see whether antisense inhibition of p65 expression reduced the rate of cell proliferation. When HL-60 cells in culture were treated with the anti-*myc* oligomer, dose-dependent inhibition of proliferation was found, with a roughly linear plot indicating 50% inhibition of proliferation at ≈4 μM (Fig. 5). However, exposure of HL-60 cells to either the anti-VSV or anti-*tat* oligomer had no effect on their proliferation.

To further test the specificity of inhibition, avian cells transformed by an avian v-*myc* gene were tested for their susceptibility to the human anti-c-*myc* oligomer. The quail embryo fibroblast line Q8 transformed by the replication-defective avian myelocytomatosis virus MC29 (24) was treated with both the anti-c-*myc* and the anti-VSV oligodeoxynucleotides. The v-*myc* gene in MC29 displays 4 mismatches in the 15 nucleotides corresponding to the target sequence in human c-*myc*, as does the chicken c-*myc* sequence (34). Neither oligodeoxynucleotide elicited any inhibition of proliferation (data not shown).

## DISCUSSION

It is initially surprising that unprotected oligodeoxynucleotides could enter a cell and remain intact long enough to have an impact, as described above. Now that several laboratories have observed the phenomenon (12–19), it is necessary to study the mechanism of uptake, compartmentalization of the oligodeoxynucleotides, and their metabolism. One possible explanation is that oligodeoxynucleotides bind nonspecifically to cell membranes and are internalized slowly in the course of normal membrane turnover. However, the observation of receptor-mediated binding of bacteriophage λ DNA to human leukocytes (35), with subsequent endocytosis and degradation to oligodeoxynucleotides, allows the possibility of oligodeoxynucleotide binding to specific receptors. For

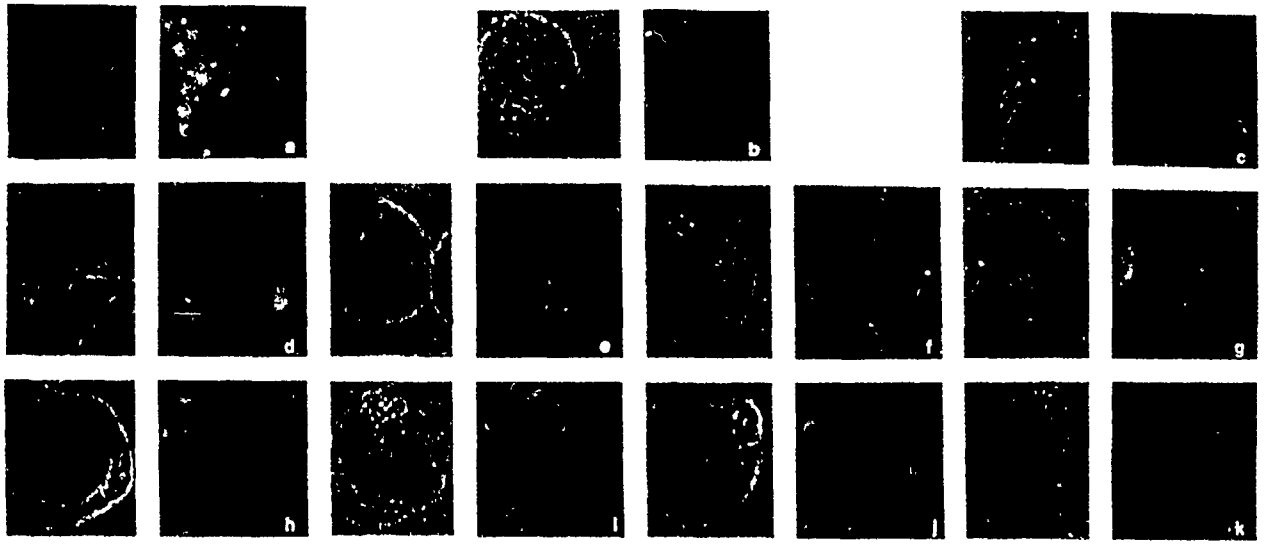

FIG. 3. Inhibition of human c-*myc* p65 protein expression by antisense oligodeoxynucleotides, measured by indirect immunofluorescence. HL-60 cells were treated with oligodeoxynucleotides for 6 hr, then immunolabeled with rabbit-produced antibodies, followed by goat-produced anti-rabbit IgG conjugated with fluorescein, and observed by light and fluorescence microscopy. (*a*) No oligodeoxynucleotide, anti-β-actin antibody. (*b*) No oligodeoxynucleotide, normal rabbit IgG. (*c*) No oligodeoxynucleotide, anti-p65 antibody. (*d*) Anti-VSV oligomer (6 μM), anti-β-actin antibody. (*e*) Anti-VSV oligomer (10 μM), anti-β actin antibody. (*f*) Anti-c-*myc* oligomer (6 μM), anti-β-actin antibody. (*g*) Anti-c-*myc* oligomer (10 μM), anti-β-actin antibody. (*h*) Anti-VSV oligomer (6 μM), anti-p65 antibody. (*i*) Anti-VSV oligomer (10 μM), anti-p65 antibody. (*j*) Anti-c-*myc* oligomer (6 μM), anti-p65 antibody. (*k*) anti-c-*myc* oligomer (10 μM), anti-p65 antibody. (×1600).

example, fluorescently labeled oligodeoxynucleotides have been used to observe rapid binding to cell membranes, which was complete within 5 min (14). It is clearly necessary to determine whether oligodeoxynucleotide receptors exist, the distribution of oligodeoxynucleotides among cytoplasm, endoplasmic reticulum, lysosomes, Golgi apparatus, mitochondria, and nuclei; and the relative rates of oligodeoxynucleotide degradation in each compartment.

In the absence of data from nuclease mapping, chemical probing, base-pair replacement, or phylogenetic comparisons, secondary-structure predictions are often misleading (36). The free energies used for base pairing are still approximate, and we lack algorithms to predict tertiary-structure interactions. Nevertheless, calculation of an oncogene mRNA secondary structure provides a starting point for selecting initial targets for hybridization arrest with antisense oligodeoxynucleotides. The effectiveness of the anti-c-*myc* oligomer predicted above at inhibiting c-*myc* protein expression and transformed-cell proliferation suggests that calculation of mRNA secondary structures may be useful in this regard.

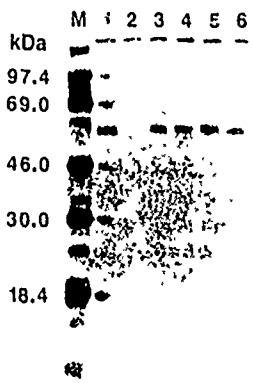


FIG. 4. Inhibition of human c-*myc* p65 protein expression by antisense oligodeoxynucleotides, measured by immunoprecipitation. HL-60 cells treated with oligodeoxynucleotides for 6 hr and then labeled with [$^{35}$S]cysteine for 1.5 hr were lysed and, immunoprecipitates were subjected to electrophoresis and fluorography. Lane M, $^{14}$C-labeled molecular mass standards, lane 1, no oligodeoxynucleotide, anti-p65 antibody, lane 2, no oligodeoxynucleotide, normal rabbit serum, lane 3, 5 μM anti-VSV oligomer, anti-p65 antibody, lane 4, 10 μM anti-VSV oligomer, anti-p65 antibody, lane 5, 5 μM anti-c-*myc* oligomer, anti-p65 antibody, lane 6, 10 μM anti-c-*myc* oligomer, anti-p65 antibody.

Indirect immunofluorescence of cells treated with antisense oligodeoxynucleotides for 6 hr showed greater reduction of p65 protein than did radioimmunoprecipitation of cells treated for 6 hr and then metabolically labeled with [$^{35}$S]cysteine for 1.5 hr. Perhaps the effective concentration of anti-c-*myc* oligomer in the cytoplasm decreases sufficiently by 6 hr to reduce the efficiency of hybrid arrest. It is also possible that a negative feedback system regulates c-*myc* transcription, leading to elevated c-*myc* mRNA levels as a result of hybrid arrest during the first 6 hr.

In view of the model that c-*myc* protein is specifically required for DNA synthesis (10), it is important to examine the impact of c-*myc* inhibition on the cell in normal human cells. Since the studies reported here on c-*myc* inhibition in

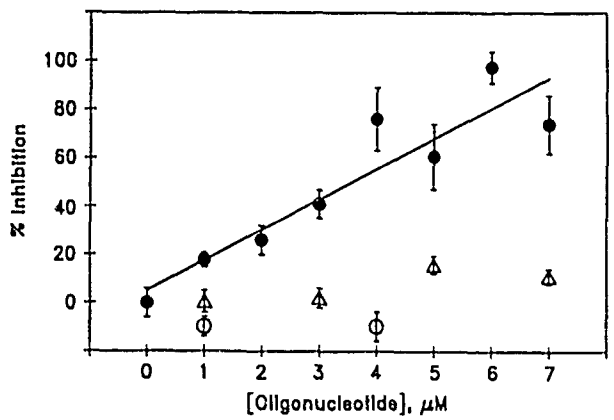


FIG. 5. Percent inhibition of HL-60 proliferation by antisense oligodeoxynucleotides. HL-60 cells were grown for 5 days in medium supplemented with anti-c-*myc* oligomer (●), anti VSV oligomer (○), or anti *tat* oligomer (△). Cells were counted and percent inhibition was calculated as described in *Materials and Methods*. Error bars on points represent one standard deviation.

HL-60 cells were begun, collaborative studies have focused on the effects of antisense oligodeoxynucleotide inhibition of c-*myc* expression in normal human peripheral blood lymphocytes (37). The anti-c-*myc* oligomer, at 30 μM, inhibited expression of c-*myc* protein, and entry into S phase, following stimulation by the mitogen phytohemagglutinin. No effect was seen with the anti-VSV oligomer, or the original sense version of the c-*myc* oligomer, or a randomly scrambled version of the anti-c-*myc* oligomer. In contrast to the HL-60 case, c-*myc* inhibition could not be detected below 15 μM anti-c-*myc* oligomer but was almost complete at 30 μM; that is, normal cells required 3 times the dose to achieve the effects seen in HL-60 cells. While the anti-c-*myc* oligomer inhibited S-phase entry, it did not prevent $G_0$ to $G_1$ traversal, consistent with the model and our results above.

Although thermodynamic calculations predicted virtually stoichiometric binding of the anti-c-*myc* oligomer to its target on c-*myc* mRNA, the concentration range of anti-c-*myc* oligodeoxynucleotide required for 50% inhibition of protein expression or cell proliferation 4–10 μM, is much higher than the concentration of c-*myc* mRNA in HL-60 cells. These cells typically contain 30–100 copies of c-*myc* mRNA per cell during logarithmic growth (J. Bresser, personal communication) and have a diameter of ≈20 μm. Cytoplasm represents only about 20% of HL-60 cell volume, so we calculate a cytoplasmic c-*myc* mRNA concentration of about 0.1 nM, roughly $10^{-5}$ of the effective anti-c-*myc* oligomer concentration.

Given these results, it is now necessary to elucidate the actual mechanism of anti-c-*myc* oligomer inhibition of HL-60 proliferation and c-*myc* protein expression, which could be at the level of mRNA transcription, processing, translation, or degradation, and to identify the factors that reduce the predicted effectiveness of the antisense oligodeoxynucleotide, including degradation, uptake, intracellular diffusion, compartmentalization, and competition with ribosomes and initiation factors. Further probing with oligodeoxynucleotides complementary to other sites in the c-*myc* mRNA and with oligodeoxynucleotides of varying complementarity to the first 5 codons may better identify accessible portions of c-*myc* mRNA and the sequence-specificity requirements of c-*myc* inhibition.

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# ANTI-C-*MYC* DNA INCREASES DIFFERENTIATION AND DECREASES COLONY FORMATION BY HL-60 CELLS

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## SUMMARY

The proto-oncogene c-*myc*, whose gene product has a role in replication, is overexpressed in the human promyelocytic leukemia HL-60 cell line. Treatment of HL-60 cells with an antisense oligodeoxyribonucleotide complementary to the start codon and the next four codons of c-*myc* mRNA has previously been observed to inhibit c-*myc* protein expression and cell proliferation in a sequence-specific, dose-dependent manner. Comparable effect are seen upon treatment of HL-60 cells with dimethylsulfoxide ($Me_2SO$), which is also known to induce granulocytic differentiation of HL-60 cells. Hence, the effects of antisense oligomers on cellular differentiation were examined and compared with $Me_2SO$. Differentiation of HL-60 cells into forms with granulocytic characteristics was found to be enhanced in a sequence-specific manner by the anti-c-*myc* oligomer. No synergism was observed between the anti-c-*myc* oligomer and $Me_2SO$ in stimulating cellular differentiation. In contrast, synergism did appear in the inhibition of cell proliferation. Finally, the anti-c-*myc* oligomer uniformly inhibited colony formation in semisolid medium. It is possible that further reduction in the level of c-*myc* expression by antisense oligomer inhibition may be sufficient to allow terminal granulocytic differentiation and reverse transformation.

*Key words:* oligodeoxynucleotides; hybrid arrest; proliferation; granulocytes; dimethylsulfoxide.

## INTRODUCTION

Overexpression of the human proto-oncogene c-*myc* has been observed frequently in human leukemias and solid tumors (1,2). The c-*myc* p65 antigen is localized in the nucleus (3), and overexpressed p65 promotes replication of SV40 DNA (4). Furthermore, antibodies against p65 coprecipitate mammalian origins of replication (5). Hence, it seems that the c-*myc* gene product plays a direct or indirect role in replication.

The HL-60 cell line, which consists predominantly of rapidly dividing cells with promyelocytic characteristics (6), contains multiple copies of the c-*myc* gene (7), and expresses 35–100 copies of c-*myc* mRNA per cell, compared with the normal 5–10 copies per cell (J. Bresser, personal communication). Dimethyl sulfoxide ($Me_2SO$) inhibits the proliferation of this cell line and induces the cells to differentiate into granulocytic cells that exhibit morphologic and chemical properties similar to more mature myelocytes, metamyelocytes, and banded and segmented granulocytes (6). Phorbol 12-myristate 13-acetate (PMA) similarly inhibits HL-60 cell proliferation and induces differentiation along the monocytic line (8).

Coincidentally, inducing HL-60 cells to differentiate with $Me_2SO$ leads to a decline in c-*myc* mRNA (9). Furthermore, it is clear that constitutive overexpression of c-*myc* in mouse erythroleukemia cells (10), or v-*myc* in human U-937 monoblastic cells (11), blocks induction of differentiation by $Me_2SO$ or PMA, respectively. Hence, it is possible that reducing the level of c-*myc* mRNA translation by antisense oligomer inhibition may be sufficient to allow differentiation and reverse transformation, similarly to $Me_2SO$.

Calculation of a predicted secondary structure for human c-*myc* mRNA placed the start codon at the beginning of a large bulge loop in a weakly base paired region, suggesting that these codons might be readily available for hybridization arrest (12). In preliminary experiments utilizing an antisense oligomer against the predicted initiation codon loop, sequence-specific,

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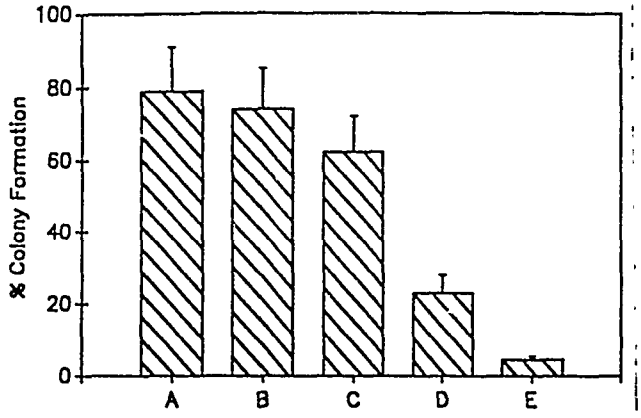


FIG. 6. Percentages of HL-60 cells forming colonies in semisolid medium treated with $Me_2SO$, PMA, or antisense oligomers. *A*, untreated; *B*, 5 $\mu M$ anti-VSV oligomer; *C*, 5 $\mu M$ anti-c-*myc* oligomer; *D*, 1% $Me_2SO$; *E*, 16 n*M* PMA.

the cells to a semisolid medium so that dividing cells will form colonies (19). HL-60 cells treated with a single dose of 5 $\mu M$ anti-c-*myc* oligomer exhibited a sequence-specific decrease in colony formation after 5 d of growth (Fig. 5). Colony size also decreased significantly, down to two to four cell colonies, and the effect was uniform throughout the culture; no resistant subpopulation was observed. In contrast, no significant decrease in colony number or size was noted in cells treated with 5 $\mu M$ anti-VSV oligomer, whereas cells treated with $Me_2SO$ and PMA created few colonies (none larger than four cells).

Upon counting colonies and cells in each cell well, it was found that 79 ± 12% of the untreated cells formed colonies, while 74 ± 11% of cells treated with the anti-VSV oligomer continued to form colonies (Fig. 6). However, colony formation by cells treated with anti-c-*myc* oligomer was reduced to 62 ± 10%. In contrast, only 23 ± 5% of cells treated with $Me_2SO$ formed colonies, and only 4 ± 1% of those treated with PMA.

## DISCUSSION

Both $Me_2SO$ and antisense oligomers inhibit c-*myc* expression, and it now seems that either mode of c-*myc* inhibition allows some degree of granulocytic differentiation and inhibition of colony formation to occur in HL-60 cells. The lack of synergism between the two agents in stimulating differentiation suggests that they may operate by different mechanisms. Furthermore, the existence of synergism where antiproliferation is concerned suggests that $Me_2SO$ may exert pleiotropic effects on replication, more than just reducing the level of c-*myc* p65. Finally, the uniform inhibition of colony formation in semisolid medium implies the effects of the anti-c-*myc* oligomer are felt by the entire HL-60 culture and that there is no resistant subpopulation.

Although $Me_2SO$ is too toxic for administration in animals at a dose of 1% by volume, the anti-c-*myc* oligomer at the concentrations used here did not inhibit c-*myc* expression in mitogen-stimulated peripheral blood lymphocytes and was not noticeably toxic to them (13). Hence, antisense inhibitors may have therapeutic potential as inducers of differentiation. It is clearly necessary to try adding antisense DNA inhibitors to cells every day, rather than only once at the beginning of the experiment, and to examine the morphologic, histochemical, and surface marker effects of the added oligomers every day.

Previous studies of oligomer degradation, uptake by cells, and survival within cells showed that oligomer degradation was complete within 15 min in undiluted FBS at 37° C (20), within 8 h in the culture medium used here RPMI 1640 with 10% heat-inactivated FBS, and was about three quarters complete within 24 h for oligomers taken up by HL-60 cells (14). Given these results, it would be worthwhile to try antisense oligomer inhibition in a serum-free medium, or with nuclease-resistant oligodeoxynucleotide derivatives.

Further probing with oligomers complementary to other sites in the c-*myc* mRNA, and with oligomers of varying complementarity to target sequences, may better identify accessible portions of c-*myc* mRNA and the sequence-specificity requirements of antisense inhibition. Finally, several other cell lines that overexpress c-*myc* should be studied for their susceptibility to antisense inhibitors of mRNA translation.

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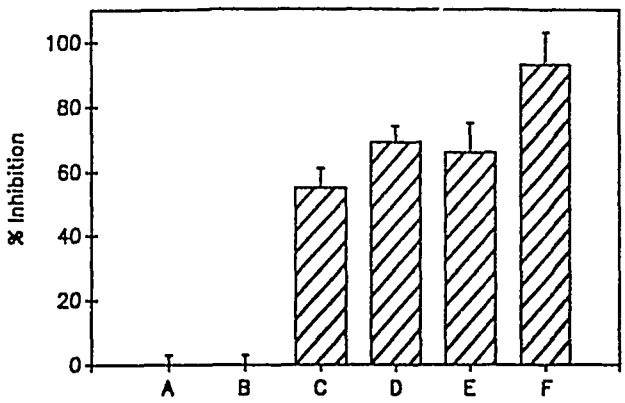


FIG. 4. Percent inhibition of HL-60 cell proliferation by combinations of antisense oligomers and $Me_2SO$. *A*, untreated; *B*, 4 $\mu M$ anti-VSV oligomer; *C*, 4 $\mu M$ anti-c-*myc* oligomer; *D*, 1% $Me_2SO$; *E*, 1% $Me_2SO$ plus 4 $\mu M$ anti-VSV oligomer; *F*, 1% $Me_2SO$ plus 4 $\mu M$ anti-c-*myc* oligomer.

3% in treated cells (Fig. 2). No cells with monocytic characteristics were observed. However, 4 $\mu M$ anti-c-*myc* oligomer was not nearly as effective at inducing differentiation as 1% $Me_2SO$, which gave 78 ± 6% differentiated cells, as seen before (6), and anti-c-*myc* oligomer does not seem to potentiate the induction of differentiation associated with $Me_2SO$. A negative control sequence against the VSV matrix protein mRNA had no effect on differentiation.

*Cytochemical analysis.* A high level of NBT reduction correlates with terminally differentiating granulocytic cells, a low level of NBT reduction correlates with immature differentiating cells, while the complete lack of NBT reduction is associated with monocytic differentiation in HL-60 cells (18). Accordingly, untreated HL-60 cells were 33 ± 5% positive for NBT reduction after 2 d, as were cells treated with 5 $\mu M$ anti-VSV oligomer, whereas cells treated with 5 $\mu M$ anti-c-*myc* oligomer rose to 48 ± 7% positive, and cells treated with $Me_2SO$ rose to 65 ± 8% (Fig. 3). In contrast, cells treated with PMA decreased to less than 5 ± 1% positive. The uninduced HL-60 cells used in these and subsequent experiments (Bacon et al., unpublished results) regularly displayed levels of NBT-positive cells in the range of 30%, by our criteria, whereas the $Me_2SO$-induced cells attained high levels similar to those reported by Collins et al. (18).

*Inhibition of cell proliferation.* Exposure of HL-60 cells to 1% $ME_2SO$ resulted in 69 ± 3% inhibition of proliferation (Fig. 4), as seen before (6), comparable to the inhibition of proliferation at 6 $\mu M$ anti-c-*myc* oligomer which was seen previously (4). Treatment of HL-60 cells with 4 $\mu M$ anti-c-*myc* oligomer reduced cell proliferation by 55 ± 5%, whereas the combination of 4 $\mu M$ anti-c-*myc* oligomer with 1% $Me_2SO$ reduced cell proliferation by 93 ± 10%. Hence, the two reagents seem to have complementary inhibitory activity toward proliferation. In a negative control experiment, the anti-VSV oligomer was not inhibitory by itself, nor did it potentiate the effect of 1% $Me_2SO$.

*Colony formation in semisolid medium.* A second method for evaluating the growth potential of a cell line is to add

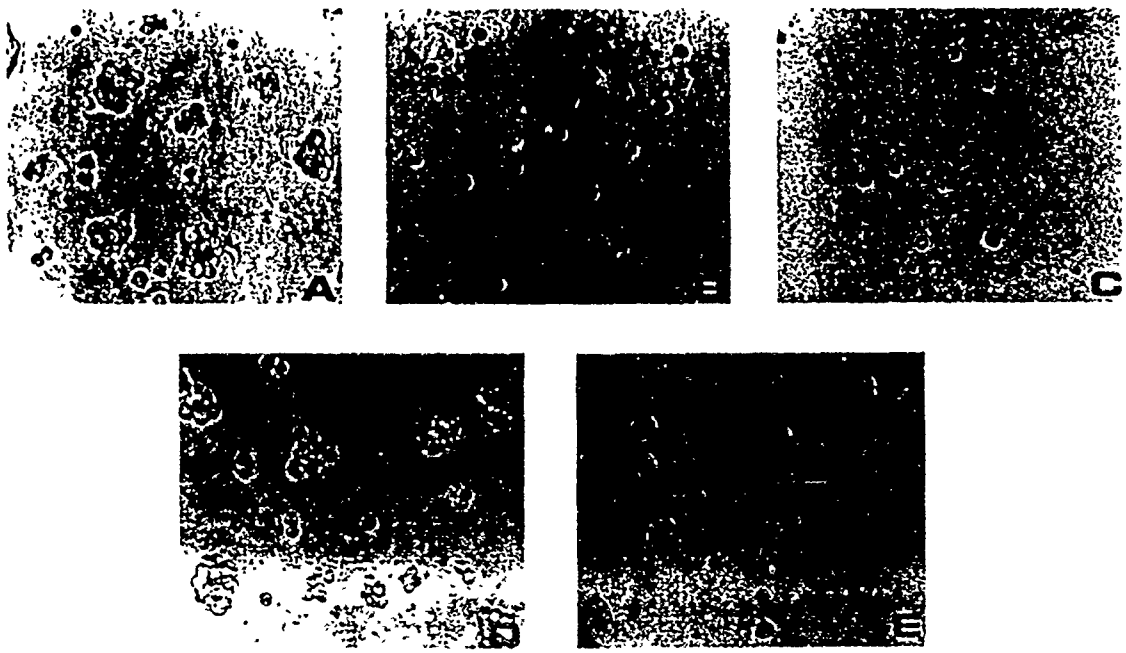


FIG. 5. Colony formation by HL-60 cells in semisolid medium treated with $Me_2SO$, PMA, or antisense oligomers. *A*, untreated; *B*, 1% $Me_2SO$; *C*, 16 n*M* PMA, *D*, 5 $\mu M$ anti-VSV oligomer; *E*, 5 $\mu M$ anti-c-*myc* oligomer.

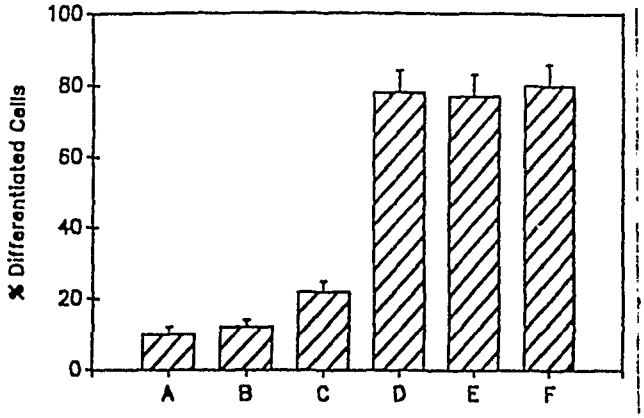


FIG. 2. Differential counts of HL-60 cell populations treated with antisense oligomers or $Me_2SO$ or both. *A*, untreated; *B*, 4 $\mu M$ anti-VSV oligomer; *C*, 4 $\mu M$ anti-c-*myc* oligomer; *D*, 1% $Me_2SO$; *E*, 1% $Me_2SO$ plus 4 $\mu M$ anti-VSV oligomer; *F*, 1% $Me_2SO$ plus 4 $\mu M$ anti-c-*myc* oligomer.

forms with a more mature appearance, including a smaller size, lower nuclear:cytoplasmic ratio, less prominent cytoplasmic granules, reduction or disappearance of nucleoli, and marked indentation, convolution, or segmentation of the nuclei. Cells in this category were scored as differentiated (6). Cells with monocytic characteristics are not observed in uninduced HL-60 cells nor those induced with $Me_2SO$, but only among cells induced with PMA. The percent differentiation in 200 cells counted was calculated from (differentiated cells/200) $\times$ 100, $\pm$ SE.

*Cytochemical analysis.* Nitro-blue tetrazolium (NBT) is reduced intracellularly to an insoluble formazan dye by superoxide, which is generated by phagocytosis-associated oxidative metabolism in normal granulocytes (17) and $Me_2SO$-differentiated HL-60 cells (18), which have been exposed very briefly to PMA. Hence, cells treated with oligodeoxynucleotides were grown for 2 d, and then 400-$\mu$l aliquots of cell suspensions were added to an equal volume of 0.2% NBT (Sigma) in calcium-and magnesium-free phosphate buffered saline, and incubated for 20 min in the presence of 160 n*M* PMA, precisely as described (17). The cells were sedimented onto glass slides and Wright-Giemsa counterstained as described above. Cells containing any reduced blue-black formazan dye were scored as NBT positive. The percent NBT-positive cells in 200 counted was calculated from (NBT positive cells/200) $\times$ 100 $\pm$ SE.

*Cell proliferation.* Treated and untreated cells showed 98-100% viability after 5 d growth, and untreated cells typically showed a 10-fold greater titer, about $10^6$ cells/ml. Cell counts were converted to percent inhibition by using the equation: $100 \times (N_n\text{-}N)/(N_n\text{-}N_0)$, where $N_0$ is the normal titer at the beginning of the experiment, $N_n$ is the titer for untreated cells after $n$ days growth, and $N$ is the titer for treated cells after $n$ days. Error bars on points represent one SD.

Semisolid medium was prepared by adding 10 g of 4000 mPa methylcellulose (Fluka) to 250 ml of sterile boiling $H_2O$, adding 250 ml of 2X RPMI 1640 with 20% bovine serum albumin BSA and stirring overnight at 0-4° C (19).

For experiments in semisolid media, aliquots of HL-60 cells (<10 $\mu$l) containing $10^4$ cells were diluted to 1 ml in semisolid RPMI 1640 with 10% FBS containing no addition, 1% $Me_2SO$, 16 nM PMA, anti-c-*myc* oligomer, or anti-VSV oligomer at concentrations detailed in the text. Cells were visualized microscopically and colonies of two or more cells were counted and compared with single cell counts. Percentages of cells forming colonies were determined daily by counting 200 colonies/cells in the same quadrant of the well.

## RESULTS

*Morphologic analysis.* HL-60 cells induced by $Me_2SO$ to differentiate with granulocytic characteristics (6) (Fig. 1 *B*), and cells induced by PMA to differentiate with monocytic characteristics (8) (Fig. 1 *C*), were compared with cells that were induced by the anti-c-*myc* and anti-VSV oligomers. Oligomers were added to a concentration of 5 $\mu M$ because previous work indicated that the antigen inhibition and antiproliferative responses were about half maximal in the 4-6 $\mu M$ range (14). Cells treated with 5 $\mu M$ anti-VSV oligomer (Fig. 1 *D*) were as undifferentiated as untreated cells (Fig. 1 *A*), but 5 $\mu M$ anti-c-*myc* oligomer induced some differentiation along the granulocytic line (Fig. 1 *E*).

Differential counts of treated cells demonstrated that 4 $\mu M$ anti-c-*myc* oligomer, the concentration which inhibited cell proliferation by 50% (14), doubled the fraction of differentiated cells resembling granulocytes from 10 $\pm$ 2% in untreated cells to 22 $\pm$

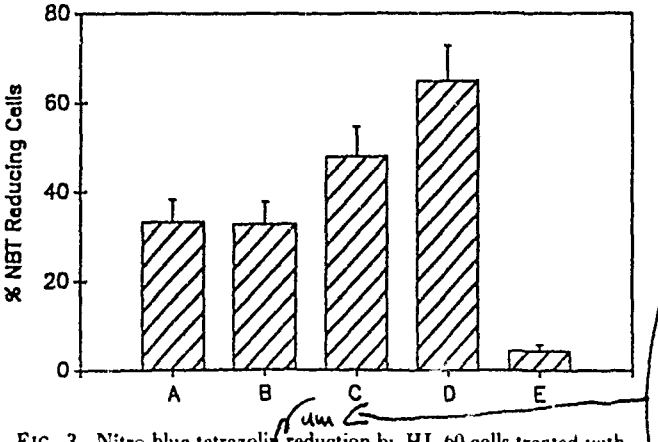


FIG. 3. Nitro-blue tetrazolium reduction by HL-60 cells treated with $Me_2SO$, PMA, or antisense oligomers. *A*, untreated; *B*, 5 $\mu M$ anti-VSV oligomer; *C*, 5 $\mu M$ anti-c-*myc* oligomer; *D*, 1% $Me_2SO$; *E*, 16 n*M* PMA.

dose-dependent inhibition of proliferation and stimulation of granulocytic differentiation were observed in human HL-60 promyelocytic leukemic cells.

Subsequently it was found that our original anti-c-*myc* oligomer also inhibited expression of the c-*myc* p65 antigen in mitogen-stimulated human peripheral blood lymphocytes, which were then unable to enter S phase in a sequence-specific, dose-dependent manner (13). The same anti-c-*myc* oligomer was roughly 3 times as effective against human HL-60 promyelocytic leukemic cells (14). Indirect immunofluorescence analysis of HL-60 cells treated with a single dose of 6 $\mu M$ anti-c-*myc* oligomer showed substantial reduction in the level of nuclear p65 protein. Inhibition of HL-60 cell proliferation by a single dose reached 50% at approximately 4 $\mu M$.

The HL-60 antiproliferation results have been confirmed by Holt et al. (15), who also reported myeloid differentiation of the treated cells. Similarly, Yokoyama and Imamoto (16) induced antisense c-*myc* RNA expression in HL-60 cells, and reported cytochemical data implying monocytic differentiation characteristics.

In the studies reported here, treatment of HL-60 cells with the anti-c-*myc* oligomer, which was previously found to depress p65 expression, elicited a sequence-specific induction of myeloid differentiation with granulocytic characteristics, potentiated inhibition of cell proliferation by $Me_2SO$, and uniformly inhibited colony formation in semisolid medium.

## Materials and Methods

*Cell culture in suspension.* HL-60 cells were grown at 37° C in 5% $CO_2$ saturated with water and maintained in logarithmic phase as before (14), in RPMI 1640 medium (Sigma, St. Louis, MO) with 10% heat inactivated fetal bovine serum (FBS, Sigma), with 1000 U/ml of penicillin, 100 $\mu$g/ml of streptomycin, and 500 $\mu$g/ml of gentamicin. Titers of viable cells were determined by counting Trypan blue excluding cells in a hemacytometer. The anti-c-*myc* oligomer, 5′-dAACGTTGAGGGGCAT-3′, and the anti-VSV oligomer, 5′-dTTGGGATAACACTTA-3′, were synthesized and purified as before (14). The cells were diluted in 1 ml of culture medium to $10^5$ cells/ml, and $Me_2SO$ (Sigma), PMA (Sigma), or oligomers or both were then added directly to cell suspensions at concentrations described below.

*Morphologic analysis.* Treated cells were grown for 5 d, and then 400–600-$\mu$l aliquots of cell suspensions were sedimented onto glass slides using a Shandon Cytospin I (Surrey, England) and Wright-Giemsa stained for differential counts performed under light microscopy (6). HL-60 cells consist predominantly of promyelocytes with large round nuclei, prominent nucleoli, dispersed nuclear chromatin, high nuclear:cytoplasmic ratio, and basophilic cytoplasm with prominent azurophilic granules. Cells in this category and cells actively undergoing mitosis were scored as undifferentiated. A moderate percentage of the uninduced cells and a large percentage of $Me_2SO$-induced cells differentiate into


FIG. 1. Morphologic differentiation of HL-60 cell populations treated with $Me_2SO$, PMA, or antisense oligomers. *A*, untreated, *B*, 1% $Me_2SO$, *C*, 16 n*M* PMA; *D*, 5 $\mu M$ anti-VSV oligomer; *E*, $\mu M$ anti-c-*myc* oligomer.

# Daily addition of an anti-c-*myc* DNA oligomer induces granulocytic differentiation of human promyelocytic leukemia HL-60 cells in both serum-containing and serum-free media

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Running title: HL-60 differentiation by anti-c-*myc* oligomer

Expression of the human proto-oncogene c-*myc* is necessary for replication, and may be inhibited in a sequence-specific, dose-dependent manner by an antisense oligodeoxynucleotide specific for the first five codons of c-*myc* mRNA. Antisense inhibition of c-*myc* inhibits the proliferation and enhances the differentiation of the HL-60 human promyelocytic leukemia cell line. In order to raise the efficacy of antisense oligomers, HL-60 cells were grown in a serum-free medium so as to minimize nuclease activity in the culture medium. Daily addition of anti-c-*myc* oligomer was then found to induce terminal granulocytic differentiation of 80% or more of HL-60 cells, and inhibit colony formation by ???%, comparable to 1% $Me_2SO$.

## Introduction

The human leukemic cell line called HL-60 consists predominantly of rapidly dividing cells with promyelocytic characteristics (Collins, et al., 1978). Untreated cells spontaneously differentiate (10-15%) into forms that exhibit characteristics of more mature granulocytic cells, while the promyelocytes continue to proliferate at a constant rate (Collins et al., 1977). Dimethylsulfoxide ($Me_2SO$) inhibits the proliferation of this cell line, and induces the cells to terminally differentiate into slowly proliferating granulocytic cells that exhibit morphological and chemical properties similar to more mature myelocytes, metamyelocytes and banded and segmented neutrophils; 1% $Me_2SO$ (v/v) treatment for 5 days induces 65-78% differentiation (Collins et al., 1978). Phorbol 12-myristate 13-acetate (PMA) at 16 nM, on the other hand, induces HL-60 cells to terminally differentiate within 24 hr into nonproliferating macrophage/monocytic cells that exhibit morphological and biochemical properties similar to more mature promonocytes and monocytes (Rovera et al., 1979).

Overexpression of the evolutionarily conserved proto-oncogene c-*myc* has been observed frequently in human leukemias and solid tumors (Cole, 1986; Klein and Klein, 1986; Bishop, 1987). HL-60 cells maintain multiple copies of the c-*myc* gene (Collins and Groudine, 1982), and express 35-100 copies of c-*myc* mRNA per cell, compared with the normal 5-10 copies per cell (Bresser, J., personal communication). The c-*myc* p65 antigen is localized in the nucleus (Spector, et al., 1987) and overexpressed p65 promotes replication of SV40 DNA (Classon, et al., 1987). Furthermore, antibodies against p65 have been reported to co-precipitate mammalian origins of replication (Iguchi-Ariga, et al., 1987). Hence, it appears likely that the c-*myc* gene product plays a direct or indirect role in replication.

Recently, inhibition of c-*myc* p65 expression by an antisense oligodeoxynucleotide targeted against a predicted hairpin loop containing the initiation codon of the human c-*myc* mRNA was found to inhibit mitogen-stimulated human peripheral blood lymphocytes from entering S phase (Heikkila, et al., 1987), and was observed to inhibit HL-60 cells from proliferating (Wickstrom, et al., 1988a), in a sequence-specific, dose-dependent manner. A single dose of the same anti-c-*myc* oligomer was also found to elicit a sequence-specific increase in number of HL-60 cells differentiating along the granulocytic line from the usual 10% to greater than 20% (Wickstrom, et al., 1988b). Comparable results were reported by Holt, et al. (1988)

using the identical anti-c-*myc* sequence, while Yokoyama and Imamoto (1987), using c-*myc* antisense RNA, reported differentiation along the monocytic line.

In addition, overexpression of p65 usually correlates with inability of cells to differentiate (Coppola and Cole, 1986; Schneider, et al., 1987), while induction of HL-60 cell differentiation with $Me_2SO$ coincides with a decline in c-*myc* mRNA (Westin et al., 1982) and the ability of the HL-60 cells to form colonies in semisolid medium (Filmus and Buick, 1985). While the anti-c-*myc* oligomer used so far does not potentiate differentiation by $Me_2SO$, it does appear to potentiate inhibition of cell proliferation by $Me_2SO$ (Wickstrom, et al., 1988b).

Hence, it seemed likely that reducing the level of c-*myc* mRNA translation further by daily addition of anti-c-*myc* oligomer might be sufficient to induce terminal differentiation of the entire population of *myc*-transformed cells. It was also observed that oligodeoxynucleotides are rapidly degraded, with a half-life of about two hr., in the culture medium used for growing HL-60 cells (Wickstrom, et al., 1988a), due to nucleases in fetal bovine serum (FBS) (Wickstrom, 1986). Therefore, in the studies reported here, a portion of the HL-60 cell line maintained in our laboratory was adapted to growth in a serum-free medium in order to minimize oligodeoxynucleotide degradation and maximize efficacy. The serum-dependent and serum-free cultures were both treated with antisense oligodeoxynucleotides, $Me_2SO$, and PMA, and the effects of these treatments were compared. Daily addition of the anti-c-*myc* oligomer more effectively induced granulocytic differentiation, and inhibited proliferation and colony formation, in serum-free medium than in serum-containing medium. Daily addition was much more effective than a single addition, and in the serum-free medium, was comparable in efficacy to 1% $Me_2SO$, yielding 80% or greater differentiation of the HL-60 population. The results suggest that significantly decreased levels of c-*myc* p65 protein allows the induction of terminal granulocytic differentiation in HL-60 cells.

## Results

*Development and Characterization of a Serum Free HL-60 Cell Strain*

An aliquot of the HL-60 cells growing in RPMI 1640 with 10% heat-inactivated FBS, designated HL-60-FBS, was introduced into RPMI 1640 medium supplemented with bovine serum albumin (BSA) and a mixture of insulin, transferrin, and sodium selenite (ITS), and grown continuously for over 6 months so that a steady state response to antisense oligomers could be investigated. The serum-free culture was designated HL-60-ITS.

The HL-60-ITS cells exhibited a slower growth rate than that of the parent cells, with a doubling time of 48 hr, 18 hr longer than HL-60-FBS cells, and a longer lag time before returning to logarithmic growth, two days rather than one day. Additionally, the HL-60-ITS cells displayed a 30% decrease in their level of c-*myc* protein (Fig. 1), a lower mitotic index (0.35%) (Tables 1,2), a 6% increase in differentiated cell forms of the same morphology as seen in the HL-60-FBS cells (Tables 1,2), and the same percentages of sudan black positive cells (40-50%), NBT reducing cells (30%), and naphthyl AS-D chloroacetate esterase positive cells (30%). Both strains of HL-60 cells reacted to sedimentation and resuspension in fresh medium by going into lag phase for a day, so daily additions of oligodeoxynucleotide were done by adding new aliquots of oligomer to continuous cultures, without disturbing them further.

*Antisense Inhibition of Cell Proliferation*

In agreement with our previous work (Wickstrom et al., 1988ab), sequence-specific dose-dependent inhibition of proliferation was observed after single additions of anti-c-*myc* oligomer to both the HL-60-FBS and HL-60-ITS cells, with greater inhibition noted in the HL-60-ITS cells (Fig. 2). The HL-60 cells used in the present work, the gift of Dr. Julie Djeu, did not respond as strongly to the antiproliferative effects of the anti-c-*myc* oligomer as did the culture used in the two previous studies (Wickstrom, et al., 1988ab). This characteristic probably reflects a lower copy number of c-*myc* mRNA, which varies widely among HL-60 cultures in different laboratories (Bresser, J., personal communication). No significant inhibition was noted in cells treated with anti-VSV oligomer, while cells treated with $Me_2SO$ and PMA were inhibited in their proliferation by greater than 90% (Figs. 2,3). Adherence of these normally anchorage-independent

cells was not significant in any case examined, and never exceeded 3% of the cells in culture.

HL-60-FBS cells that were treated daily for 5 days with 10 nmol/ml of anti-c-*myc* oligomer, in order to re-establish a concentration of 10 $\mu$M in the culture medium every day, exhibited a titer at day 5 similar to HL-60-FBS cells treated with a single addition of 15 $\mu$M anti-c-*myc* oligomer (Fig.2,3a) (Wickstrom et al., 1988ab). Similar results were seen with HL-60-ITS cells, with greater antiproliferative efficacy (Figs. 2,3b). No significant inhibition was noted in cells treated daily with anti-VSV oligomer, while the cells treated with a single addition of $Me_2SO$ or PMA were inhibited by greater than 90% (Figs. 2,3ab).

A second method for evaluating the growth potential of a cell line is to add the cells to a semisolid medium so that dividing cells will form colonies (Graf, et al., 1981). HL-60-FBS cells grown in semisolid medium, and treated daily with anti-c-*myc* oligomer, showed a decrease in colony formation of greater than 50%, relative to untreated cells (Fig. 4a). However, no significant decrease in colony number was noted in cells treated with anti-VSV oligomer. By contrast, 12 % or less of HL-60-FBS cells treated with $Me_2SO$ and PMA formed colonies, none greater than 2 cells. Daily treatment with antisense oligomer treatment required that the semisolid medium be stirred vigorously so that uniform distribution of the oligomer was assured. As a result, the existing colonies were significantly disturbed, and the number of colonies, and colony sizes, were reduced by at least 70%, when compared with undisturbed colonies (data not shown), even untreated HL-60-FBS cells. Similar experiments were attempted with HL-60-ITS cells, but they failed to grow beyond 36 hrs.

Upon counting colonies and cells in each cell well, it was found that 23±5% of the untreated cells formed colonies (Fig. 4b). In contrast, only 12±3% of cells treated with $Me_2SO$ formed colonies, and only 5±1% of those treated with PMA. However, colony formation by cells treated with anti-c-*myc* oligomer was reduced to 10±3%, while 20±5% of cells treated with the anti-VSV oligomer continued to form colonies.

*Morphological Analysis*

Analysis of Wright-Giemsa stained cells allows one to establish the overall degree of differentiation in a heterogeneous cell population. Untreated HL-60-FBS cells, HL-60-FBS cells induced to differentiate into mature granulocytic forms with $Me_2SO$, and into mature monocytic forms with PMA, were compared with HL-60-FBS cells treated daily with anti-c-*myc* or anti-VSV oligomer over a 5 day period. Differential counts of untreated and treated cells are shown in Table 1. Light micrographs of cells representative of each category used in scoring cell types are shown at the head of the table. Cells grown in culture at 10 $\mu$M anti-c-*myc* oligomer, replenished daily, exhibited a dramatic decrease in the number of promyelocytes and cells undergoing mitosis, a large increase in myelocytes/metamyelocytes and banded neutrophils, but little difference in segmented neutrophils or hypersegmented neutrophils,compared with untreated cells. The overall extent of differentiation into the latter four cell types was 64±10% No significant change in mature morphological forms was noted in cells treated with anti-VSV oligomer, while cells treated with $Me_2SO$ exhibited even greater differentiation into granulocytic forms, to a total of 92±13%, and cells treated with PMA exhibited virtually complete differentiation into mature monocytic forms. The time course of differentiation over 5 days is shown in Fig. 5a.

The same kind of analysis was carried out on HL-60-ITS cells, shown in Table 2 for the state of differentiation at 5 days, and the time course over 5 days appears in Fig. 5b. The most significant difference seen in the absence of serum is the greater efficacy of the anti-c-*myc* oligomer, nearly approximating that of 1% $Me_2SO$. Both in the presence and absence of serum, the predominant changes seen in Tables 1 and 2 occurred in the myelocyte/metamyelocyte category. That is, induction of differentiation by the anti-c-*myc* oligomer did not significantly drive the cells into the segmented or hypersegmented neutrophil categories.

*Cytochemical Assays*

The positive results found by morphological analysis of cells treated with daily additions of antisense oligodeoxynucleotides required confirmation by cytochemical assays for granulocytic or monocytic differentiation. Sudan black stains lipophilic granules characteristic of granulocytic differentiation, while monocytic cells stain weakly (Sheehan and Storey, 1947). HL-60-ITS cells stayed in the range of 40-50% positive for Sudan black incorporation for 5 days, as did cells treated daily with anti-VSV oligomer (Fig. 6a). On the other hand, cells treated daily with anti-c-*myc* oligomer, or with $Me_2SO$, rose to over 80% positive by 5 days, while cells treated with PMA failed to take up Sudan black at all by 2 days. Similarly striking results were seen with HL-60-FBS cells (not shown).

NBT is reduced intracellularly to insoluble formazan by superoxide, which is generated by PMA induction of phagocytosis-associated oxidative metabolism in normal granulocytes (DeChatelet et al., 1976) and differentiated HL-60 cells (Collins et al., 1979). Hence, a high level of NBT reduction correlates with terminally differentiated mature granulocytic cells, a low level of NBT reduction correlates with immature or non-differentiated cells, while the complete lack of NBT reduction is associated with monocytic or lymphocytic differentiation (Collins et al., 1979). Accordingly, untreated HL-60-ITS cells stayed in the neighborhood of 30% positive for NBT reduction over 5 days, as did cells treated daily with anti-VSV oligomer, while cells treated daily with anti-c-*myc* oligomer rose to about 60% positive by 5 days, and cells treated with $Me_2SO$ rose to about 80% (Fig. 6b). In contrast, cells treated with PMA decreased by the first day to less than 5% positive. Comparable clearcut results were seen with HL-60-FBS cells (not shown).

High levels of naphthol AS-D chloroacetate esterase activity have been observed in myeloblasts, promyelocytes, and granulocytes while little or no activity has been noted in monocytes. Conversely, high levels of $\alpha$-naphthyl acetate esterase activity have been found in monocytes but not in myeloblasts, promyelocytes, or granulocytes (Yam, et al., 1971). Hence, high levels of naphthol AS-D chloroacetate esterase correlate with immature cells and maturing cells differentiating along the granulocytic pathway while high levels of $\alpha$-naphthyl acetate esterase activity correlate with cells maturing along the monocytic pathway. Untreated HL-60-ITS cells and those treated daily with anti-VSV oligomer leveled out by three days at about 30% positive for naphthol AS-D chloroacetate esterase while those treated with anti-c-*myc* oligomer rose to about 50%

positive. In contrast, cells treated with $Me_2SO$ were over 90% positive for chloroacetate esterase by four days, while those treated with PMA displayed no detectable activity after only one day of treatment (Fig. 6c). Untreated, $Me_2SO$, anti-VSV, and anti-c-*myc* treated cells were found to be less than 2% positive for $\alpha$-naphthyl acetate esterase while PMA treated cells were greater than 90% positive (not shown). Virtually the same pattern was observed for HL-60-FBS cells (not shown).

## Discussion

The observation that HL-60 cells grew in a serum-free medium implies that growth factors in serum are not absolutely essential for their proliferation, with the possible exceptions of insulin, transferrin, and sodium selenite. It is significant, however, that the level of c-*myc*-encoded p65 decreased, and the doubling time increased, in the absence of serum. Hence, one must assume that at least some of the missing serum factors play a stimulatory role in the HL-60 cell cycle. On the other hand, the extent of differentiation was the same under both culture conditions.

The greater antiproliferative efficacy of a single addition of anti-c-*myc* oligomer in the absence of serum is consistent with the hypothesis that serum nucleases limit the amount of oligomer which may be taken up by cells over the 5 days of the experiment. In both cases, inhibition of proliferation was dose-dependent. Daily addition of anti-c-*myc* oligomer inhibited proliferation more than a single addition, consistent with the observation that oligodeoxynucleotides taken up by cells turn over within 1-2 days. The greater efficacy of daily addition was observed both in the presence and absence of serum, with an even stronger effect in the absence of serum. Comparing Figs. 2a with 3b, one sees that daily addition of 10 $\mu$M anti-c-*myc* oligomer in the absence of serum was roughly three times as effective as an antiproliferative as a single addition in the presence of serum. In no case did the control anti-VSV oligomer display any effect on proliferation, while the cells were similarly inhibited by $Me_2SO$ and PMA in the presence and in the absence of serum.

Examination of colony formation in semisolid medium provided an answer to the question of whether the HL-60 cell culture contained a subpopulation resistant to antisense inhibition of c-*myc* expression. As the concentration of daily replenished anti-c-*myc* oligomer was increased, the number of cells forming colonies decreased, and in addition the size of colonies themselves decreased. This result reaffirms the observation that c-*myc* expression was inhibited significantly in all cells following single addition of antisense oligomer (Wickstrom, et al., 1988b).

Antisense inhibition of c-*myc* gene expression in HL-60 cells grown in the presence of serum, treated with a single addition of anti-c-*myc* oligomer, has been observed to enhance differentiation along the granulocytic pathway (Wickstrom, et al., 1988b; Holt, et al., 1988). In the results presented above, the differential counts of cells treated daily with antisense oligomers provided a detailed profile of the extent of

differentiation of each population. Daily addition of 10 $\mu$M anti-c-*myc* oligomer to HL-60 cells grown without serum was found to induce terminal differentiation almost as effectively as 1% $Me_2SO$.

Cytochemical assays of HL-60 cell differentiation confirmed the morphological analyses. Sudan black staining gave the strongest response, while NBT reduction and naphthol AS-D chloroacetate activity showed a greater response to $Me_2SO$ than to anti-c-*myc* oligomer. In the latter two assays, differentiation induced by anti-c-*myc* oligomer was similar in both the presence and absence of serum. Perhaps uptake of Sudan black reaches a maximum as soon as promyelocytes enter the myelocyte/metamyelocyte stage, while superoxide and esterase levels may not maximize until the final granulocytic stages.

These observations suggest that it might be possible to keep c-*myc*-transformed leukemic cells under control by daily administration of an antisense oligomer concentration, 10 $\mu$M, which was not found to inhibit c-*myc* p65 expression in normal peripheral blood lymphocytes stimulated by phytohemagglutinin (Heikkila, et al., 1987). On the other hand, the differential counts and cytochemical assays revealed that HL-60 cells treated daily with anti-c-*myc* oligomer did not differentiate into fully mature granulocytic forms, but were found primarily in intermediate forms along the granulocytic pathway. It is worth asking whether continued antisense oligomer treatment beyond five days would differentiate the cells completely, and whether discontinuation of treatment would lead the cells to revert to promyelocytic form*s.

While it is now clear that down-regulation of c-*myc* inhibits replication, it is not at all clear how a decrease in p65 stimulates differentiation along the granulocytic pathway, as opposed to the monocytic pathway. Elucidation of this question requires examination of the impact of anti-c-*myc* oligomer treatment on both transcription and translation of a panel of developmental and cell cycle genes, in a variety of normal and transformed cell lines. It will also be useful to probe a variety of sites along the c-*myc* mRNA in order to find an optimal target for antisense inhibition, and to study efficacy as a function of oligomer length.

## Materials and methods

### *Cell Culture*

HL-60 cells were grown and maintained in log phase with greater than 85% viability in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) at 37° C in a 5% $CO_2$ atmosphere saturated with water (HL-60-FBS). A serum-free culture was established by transferring 1 ml of RPMI 1640 with 10% FBS, containing $10^6$ cells, into 9 ml of RPMI 1640 supplemented with 0.4% bovine serum albumin (BSA), 1:1000 Insulin-Transferrin-Sodium Selenite medium supplement (ITS) (Sigma) and gradually decreasing percentages of FBS over a 3 week period until cells were able to maintain greater than 80% viability without FBS (HL-60-ITS). For growth in semisolid media, the latter media also contained 2% methyl cellulose (Fluka, 4000 mP.s) (Graf, et al., 1981). All tissue culture media contained $10^5$ units of penicillin, 0.1 g streptomycin and 0.5 g gentamicin per liter. Cell titers and viability were determined by trypan blue (Gibco) dye exclusion. Error bars in cell counts represent one root-mean-square standard deviation for multiple determination, or $\sqrt{n}$ for single counts of large numbers. Adherence of cells to tissue culture plates was analyzed by washing the empty plate with warm medium, decanting, adding 0.1% trypsin for 10 min., inactivating the trypsin with an equal volume of warm medium, and counting 20 $\mu$l aliquots.

### *Radioimmunoprecipation of c-myc p65 Protein*

Samples of 2 - 3 x $10^6$ cells from the HL-60-FBS and HL-60-ITS cultures were sedimented, decanted, washed in PBS, and resuspended in 0.5 ml of cysteine-free RPMI 1640 (GIBCO) supplemented with 10% FBS or BSA-ITS and [$^{35}$S]cysteine, 1022 Ci/mmol (du Pont/New England Nuclear) at 300 $\mu$Ci/ml. Each sample was grown for an additional 1.5 hr, after which the cells were sedimented, washed, lysed, immunoprecipitated, electrophoresed, and fluorographed as described (Wickstrom, et al., 1988a).

*Cell Treatment*

HL-60 cells were sedimented, decanted, and resuspended in fresh RPMI 1640 with 10% FBS or RPMI 1640 with ITS-BSA to a concentration of $10^5$ cells/ml, and then incubated for 24 hrs in the wells of culture plates in order to re-establish logarithmic growth before addition of modulators. 1% (v/v) $Me_2SO$ (Sigma), 16 nM PMA (Sigma), anti-c-*myc* oligomer, or anti-VSV oligomer were then added directly to the suspensions at concentrations detailed in the text. The anti-VSV oligomer 5'-dTTGGGATAACACTTA-3' and anti-c-*myc* oligomer 5'-dAACGTTGAGGGGCAT-3' were prepared as before (Wickstrom et al., 1988a).

For experiments in semisolid media, aliquots of HL-60 cells (<10 $\mu$l) containing $10^4$ cells were diluted to 1 ml in semisolid RPMI 1640 with 10% FBS or semisolid RPMI 1640 with BSA-ITS. The semisolid media contained no addition, 1% $Me_2SO$ (Sigma), 16 nM PMA (Sigma), anti-c-*myc* oligomer, or anti-VSV oligomer at concentrations detailed in the text. For subsequent daily additions, the suspensions were mixed after each oligomer addition.

*Colony Formation in Semisolid Media*

Tissue culture cell wells (Corning) containing $10^4$ cells in 1 ml of semisolid RPMI 1640 with 10% FBS or semisolid RPMI 1640 with BSA-ITS media were visualized microscopically and colonies of two or more cells were counted and compared with single cell counts. Percentages of cells forming colonies were determined daily by counting 200 colonies/cells in the same quadrant of the well.

*Cell Differentiation and Mitotic Index*

Treated and control cell suspensions were thoroughly mixed, and aliquots of 200-600 $\mu$l were sedimented onto microscope slides using a Shandon Cytospin II (Surrey, England). Cell monolayers were Wright-Giemsa stained (ASP), and cell differentiation and mitotic index were determined simultaneously under light microscopy on 1000 cells. Cells scored as myeloblasts/promyelocytes and dividing cells were considered undifferentiated. Those scored as myelocytes/metamyelocytes, banded neutrophils, segmented neutrophils, and hypersegmented neutrophils were considered differentiated.

*Cytochemical Assays for Differentiation*

Cell monolayers on slides, prepared as above, were used in cytochemical assays for uptake of sudan black (Sigma) (Sheehan and Storey, 1947), reduction of nitroblue tetrazolium (NBT) (Sigma) (Collins et al., 1979), and activity of $\alpha$-naphthyl acetate esterase, and naphthol AS-D chloroacetate esterase (Sigma) (Yam et al., 1971), according to the published methods.

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**Figure 1** c-*myc* p65 protein expression of untreated HL-60-FBS and HL-60-ITS cells measured by immunoprecipitation. Cells were removed from culture on the second day of logarithmic growth, titered, labeled with [$^{35}$S]cysteine for 1.5 hr, lysed, immunoprecipitated, electrophoresed, and fluorographed. Lane M, $^{14}$C-labeled molecular mass standards; lane 1: 2 x $10^6$ HL-60-FBS cells, with rabbit IgG; lane 2: 2 x $10^6$ HL-60-FBS cells, with anti-p65 antibody; lane 3: 3 x $10^6$ HL-60-FBS cells, with anti-p65 antibody; lane 4. 2 x $10^6$ HL-60-ITS cells, with rabbit IgG; lane 5: 2 x $10^6$ HL-60-ITS cells, with anti-p65 antibody; lane 6: 3 x $10^6$ HL-60-ITS cells, with anti-p65 antibody.

**Figure 2** Titers of cells treated with a single dose of antisense oligodeoxynucleotide $Me_2SO$ or PMA in the presence or absence of serum. Cells were grown for 5 days in untreated medium (○), medium supplemented with 1% $Me_2SO$ (●), 16 nM PMA (▽), 5 μM anti-VSV oligomer (◇), 10 μM anti-VSV oligomer (□), 15 μM anti-VSV oligomer (△), 5 μM anti-c-*myc* oligomer (◆), 10 μM anti-c-*myc* oligomer (■) or 15 μM anti-c-*myc* oligomer (▲). A, with serum, HL-60-FBS cells; B, without serum, HL-60-ITS cells.

**Figure 3** Titers of cells treated with daily doses of antisense oligodeoxynucleotides, or single doses of $Me_2SO$ or PMA. Cells were grown for 5 days in untreated medium (○), medium supplemented with 1% $Me_2SO$ (●), 16 nM PMA (▽), anti-VSV oligomer (10 nmol/ml/day) (□), or anti-c-*myc* oligomer (10 nmol/ml/day) (■). A, with serum, HL-60-FBS cells; B, without serum, HL-60-ITS cells.

**Figure 4** Colony formation by HL-60-FBS cells in semisolid medium treated with daily doses of antisense oligodeoxynucleotides, or single doses of $Me_2SO$ or PMA. Cells were grown for 5 days in untreated medium (A), medium supplemented with 1% $Me_2SO$ (B), 16 nM PMA (C), anti-VSV oligomer (10 nmol/ml/day) (D), anti-c-*myc* oligomer (10 nmol/ml/day) (E). A, light micrographs of representative portions of cultures; B, percentages of cells forming colonies.

**Figure 5** Percentage differentiation along the granulocytic pathway of cells treated with daily doses of antisense oligodeoxynucleotides, or single doses of $Me_2SO$ or PMA. Cells were grown for 5 days in untreated medium (○), medium supplemented with 1% $Me_2SO$ (●), 16 nM PMA (▽), anti-VSV oligomer (10 nmol/ml/day) (□), or anti-c-*myc* oligomer (10 nmol/ml/day) (■). A, with serum, HL-60-FBS cells; B, without serum, HL-60-ITS cells.

**Figure 6** Cytochemical assays of HL-60-ITS cells treated with daily doses of antisense oligodeoxynucleotides, or single doses of $Me_2SO$ or PMA. Cells were grown for 5 days in untreated medium (○), medium supplemented with 1% $Me_2SO$ (●), 16 nM PMA (▽), anti-VSV oligomer (10 nmol/ml/day) (□), or anti-c-*myc* oligomer (10 nmol/ml/day) (■). A, Sudan black incorporation; B, NBT reduction; C, Naphthol AS-D chloroacetate esterase activity.

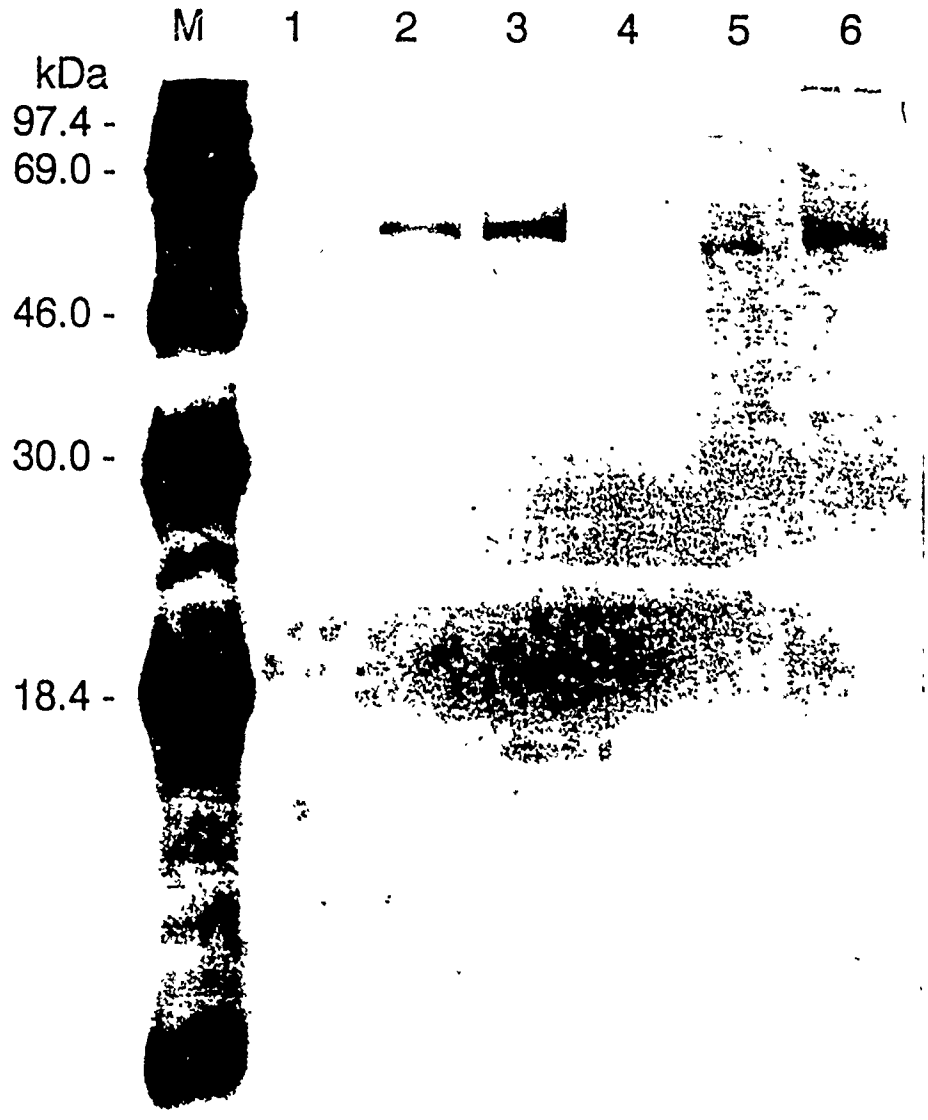

M
1
2
3
4
5
6
kDa
97.4 -
69.0 -
46.0 -
30.0 -
18.4 -

Table 1. Differential Counts of HL-60-FBS Cells After Incubation with Inducing Compounds and Antisense Deoxyoligonucleotides

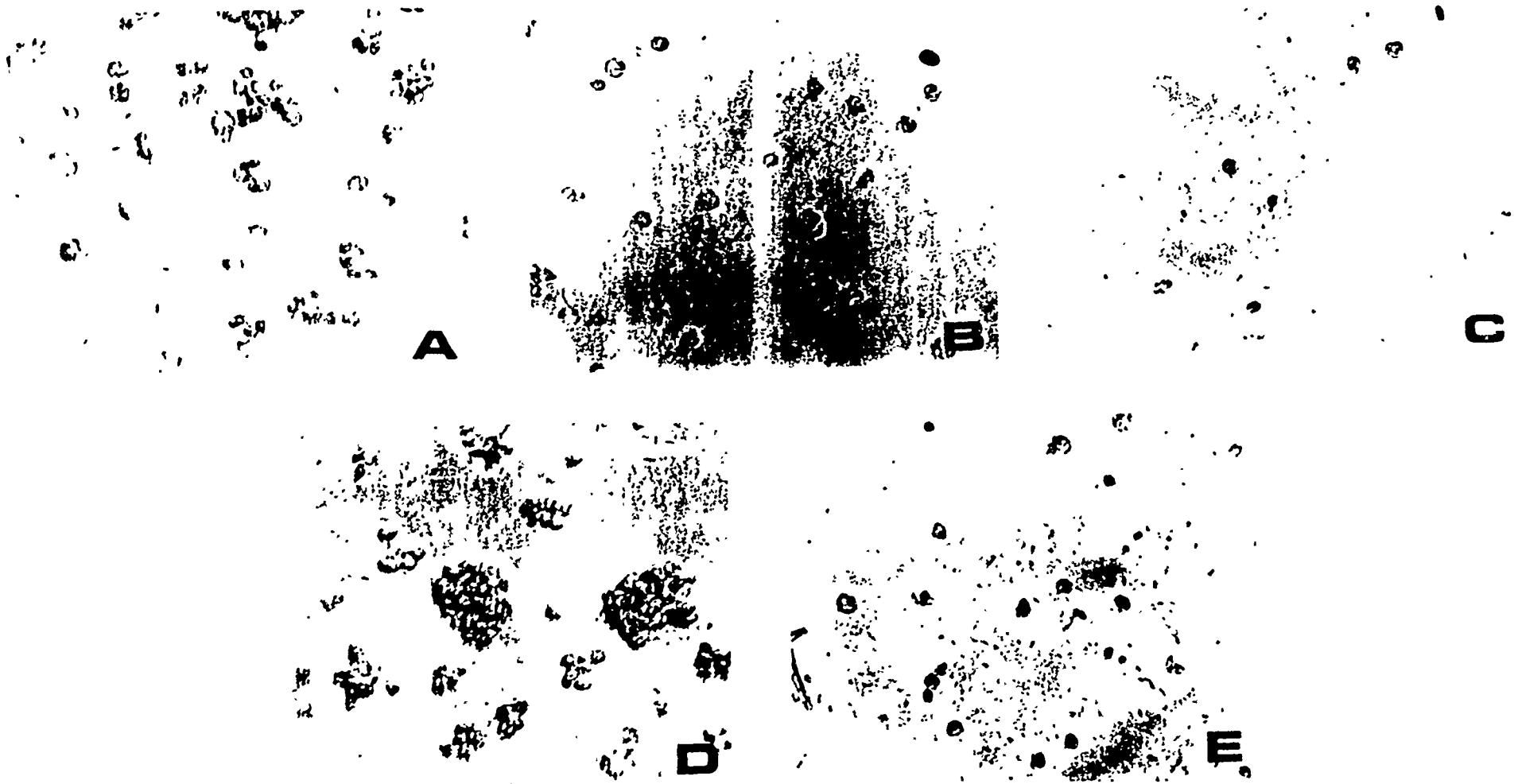
| Inducer | Mp[a] | Mb/Pm | My/Me | Ba | Sn | HSn | Mo |
|---|---|---|---|---|---|---|---|
| None (0)[b] | 20 | 888 | 46 | 5 | 37 | 4 | 0 |
| None (5)[c] | 34 | 799 | 61 | 24 | 61 | 21 | 0 |
| PMA (5)[d] | 0 | 0 | 0 | 0 | 0 | 0 | 200 |
| $Me_2SO$ (5)[c] | 5 | 70 | 806 | 21 | 72 | 26 | 0 |
| anti-VSV (5)[c] | 36 | 794 | 71 | 27 | 53 | 19 | 0 |
| anti-c-*myc* (5)[c] | 18 | 343 | 482 | 66 | 68 | 23 | 0 |

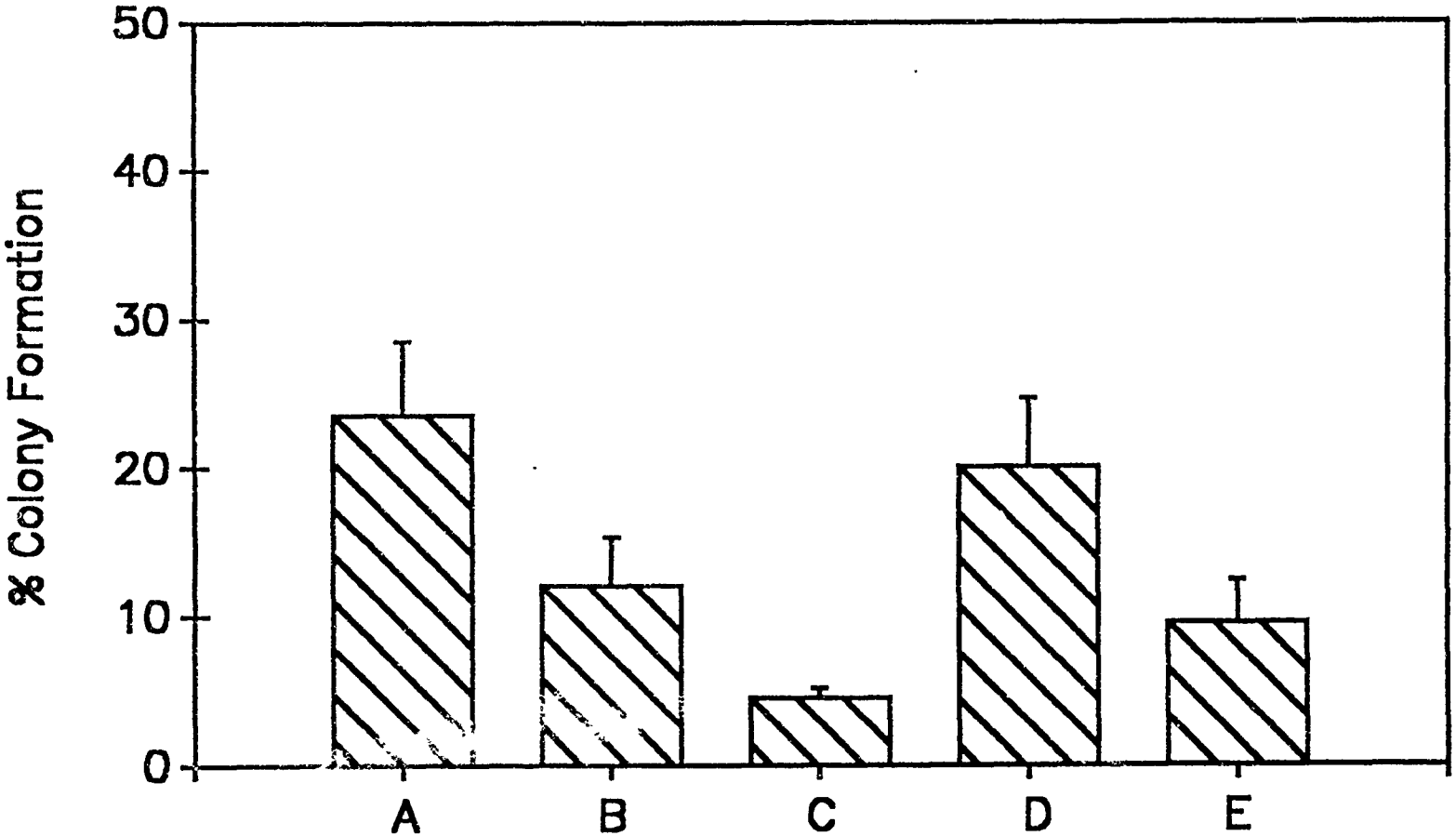
[a]Mp, Mitotic phase; Mb/Pm, myeloblasts/promyelocytes; My/Me, myelocytes/metamyelocytes; Ba, banded neutrophils; Sn, segmented neutrophils; HSn, Hypersegmented neutrophils; Mo, monocytes.
[b]Values represent the analysis of 1000 cells each from duplicate samples of one experiment on day 0.
[c]Values represent the analysis of 1000 cells from a single experiment on day 5.
[d]Values represent the analysis of 200 cells. Fewer cells were recovered after treatment with PMA due to terminal differentiation of the cells within the first 24 hrs of treatment.

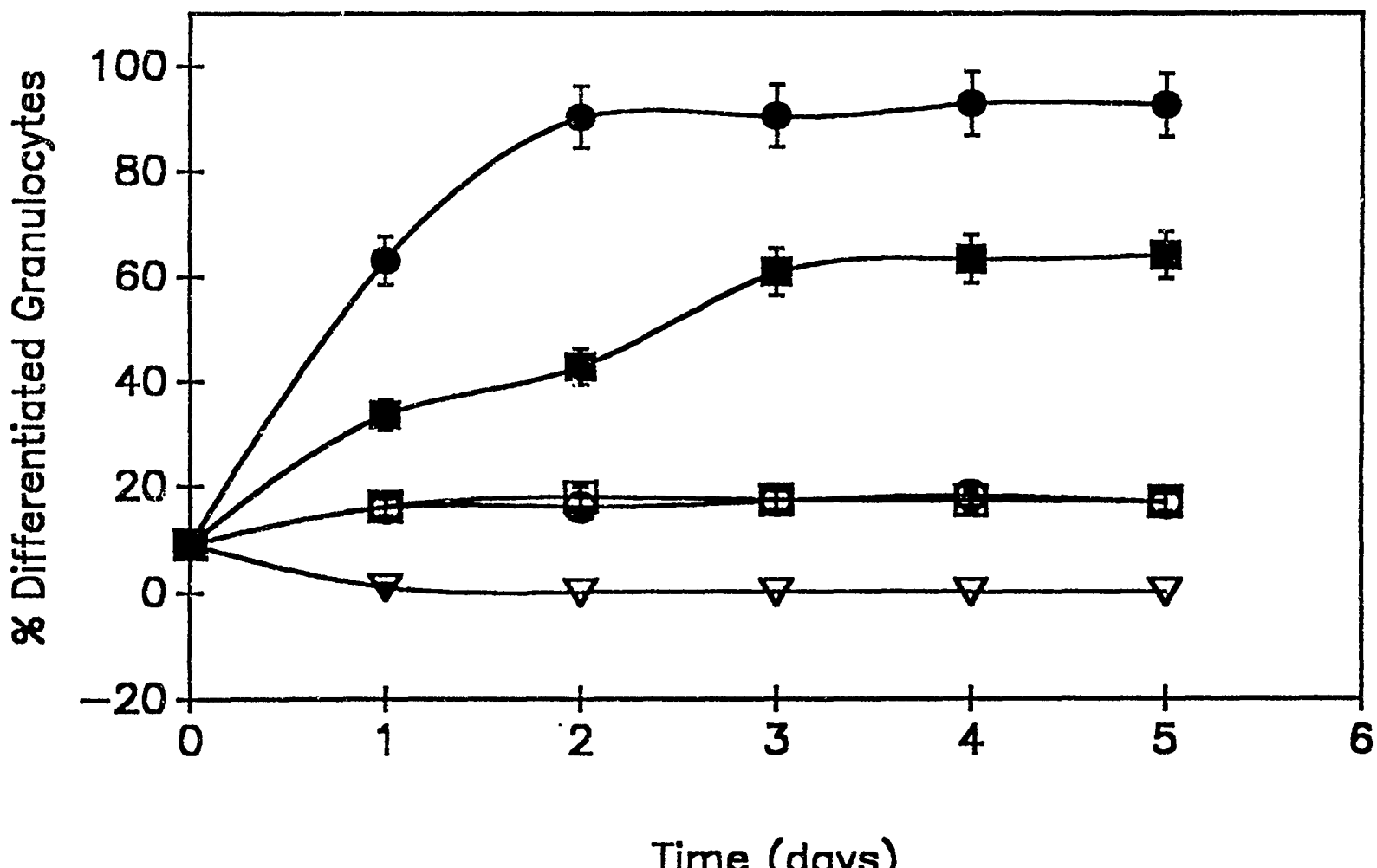
Table 2. Differential Counts of HL-60-ITS Cells After Incubation with Inducing Compounds and Antisense Deoxyoligonucleotides

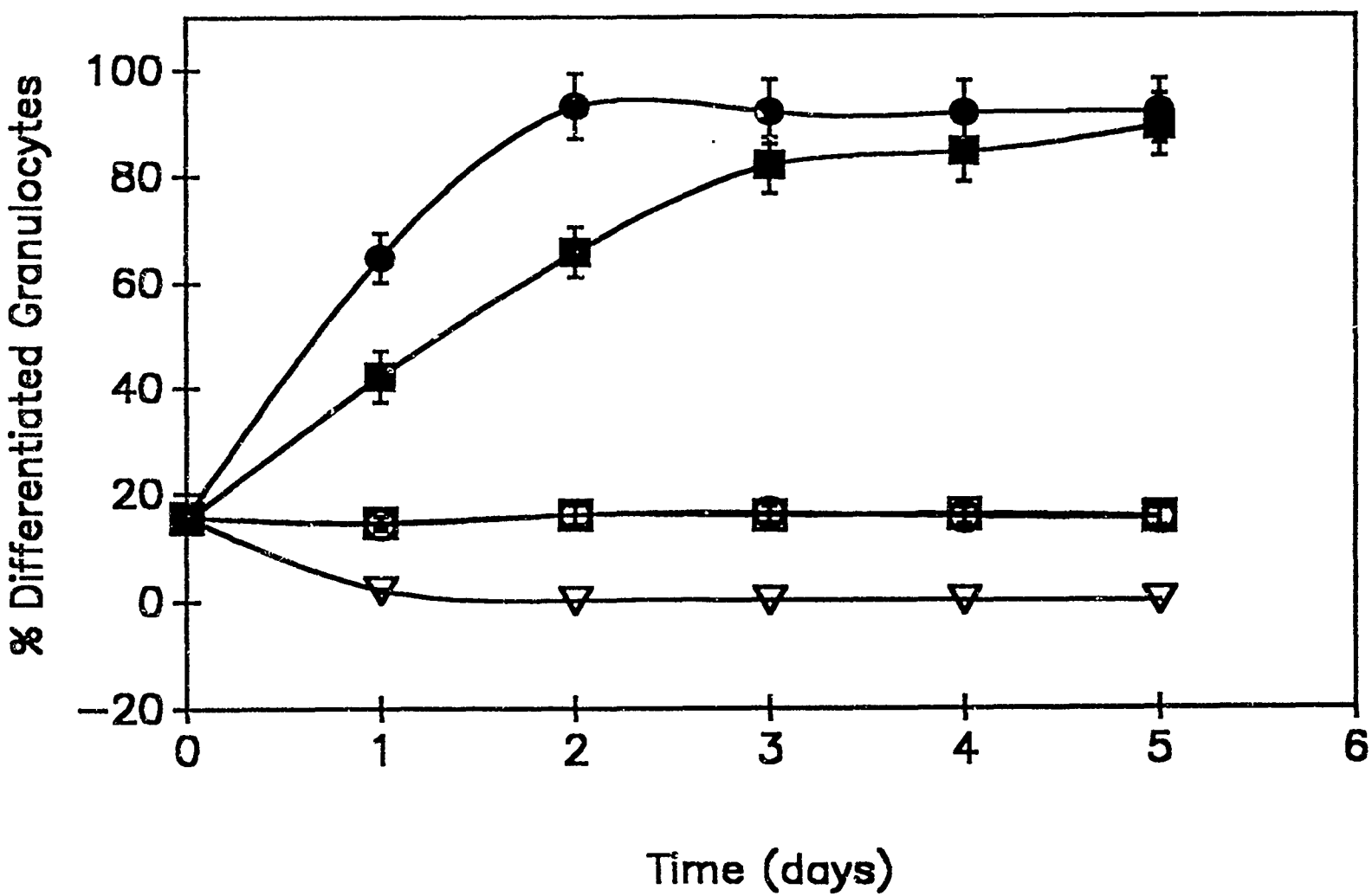
| Inducer | Mp[a] | Mb/Pm | My/Me | Ba | Sn | HSn | Mo |
|---|---|---|---|---|---|---|---|
| None (0)[b] | 17 | 826 | 55 | 17 | 69 | 16 | 0 |
| None (5)[c] | 48 | 800 | 60 | 22 | 58 | 12 | 0 |
| PMA (5)[d] | 0 | 0 | 0 | 0 | 0 | 0 | 200 |
| $Me_2SO$ (5)[c] | 18 | 61 | 781 | 18 | 74 | 28 | 0 |
| anti-VSV (5)[c] | 49 | 797 | 59 | 18 | 62 | 15 | 0 |
| anti-c-*myc* (5)[c] | 22 | 83 | 798 | 37 | 62 | 18 | 0 |

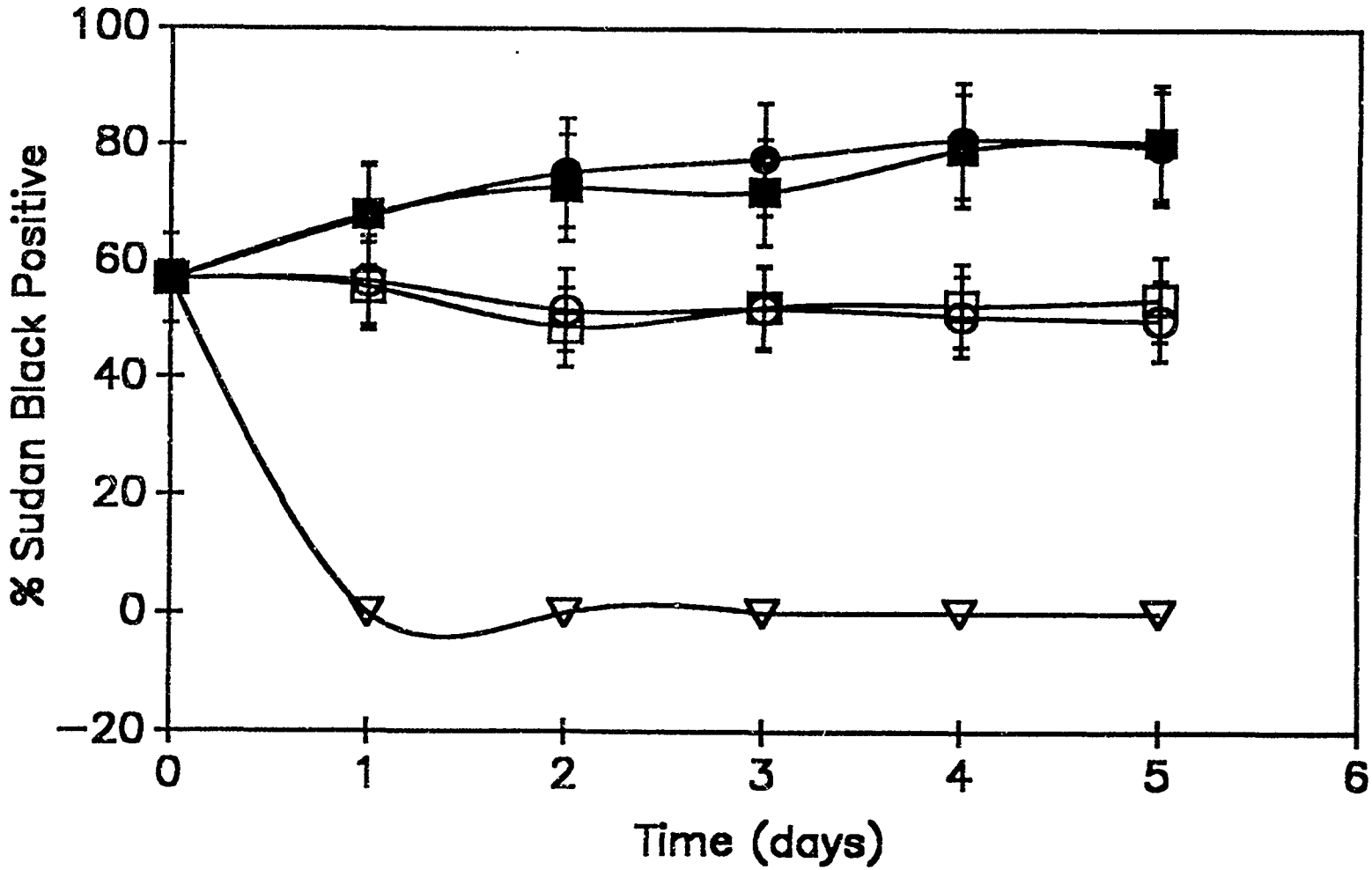
[a]Mp, Mitotic phase; Mb/Pm, myeloblasts/promyelocytes; My/Me, myelocytes/metamyelocytes; Ba, banded neutrophils; Sn, segmented neutrophils; HSn, Hypersegmented neutrophils; Mo, monocytes.
[b]Values represent the analysis of 1000 cells each from duplicate samples of one experiment on day 0.
[c]Values represent the analysis of 1000 cells from a single experiment on day 5.
[d]Values represent the analysis of 200 cells. Fewer cells were recovered after treatment with PMA due to terminal differentiation of the cells within the first 24 hrs of treatment.

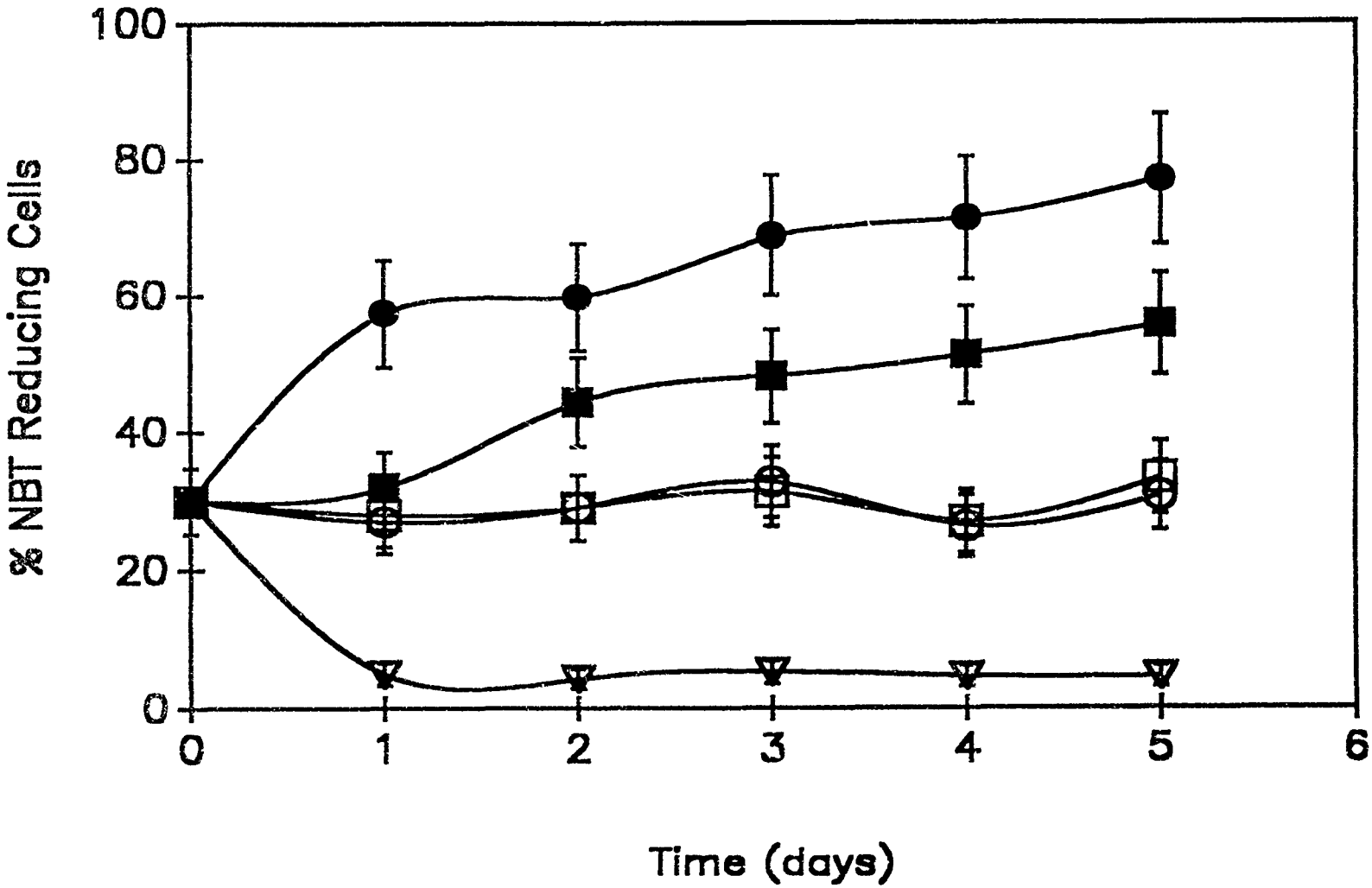

A
B
C
D
E

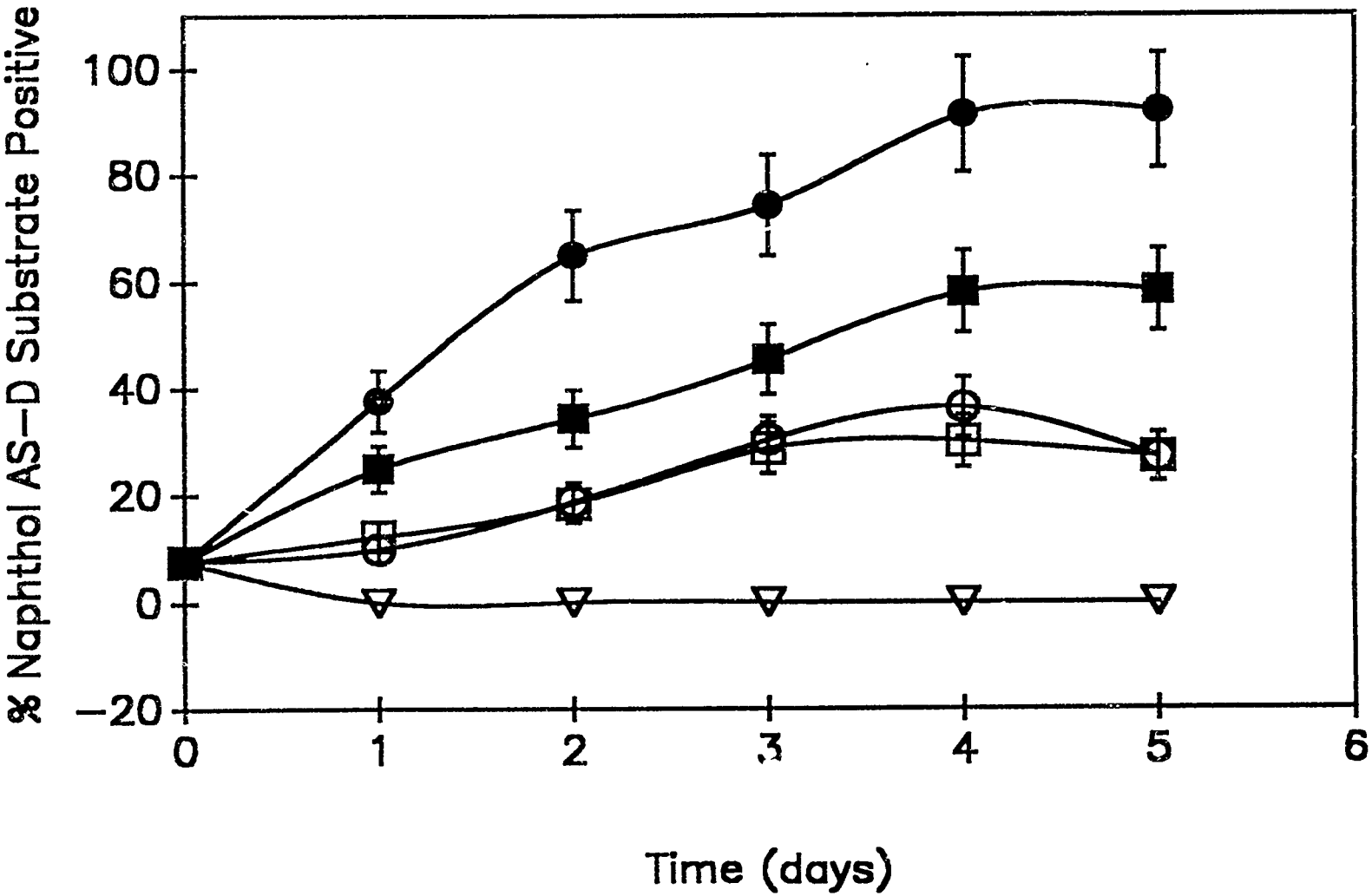

% Colony Formation
50
40
30
20
10
0
A
B
C
D
E

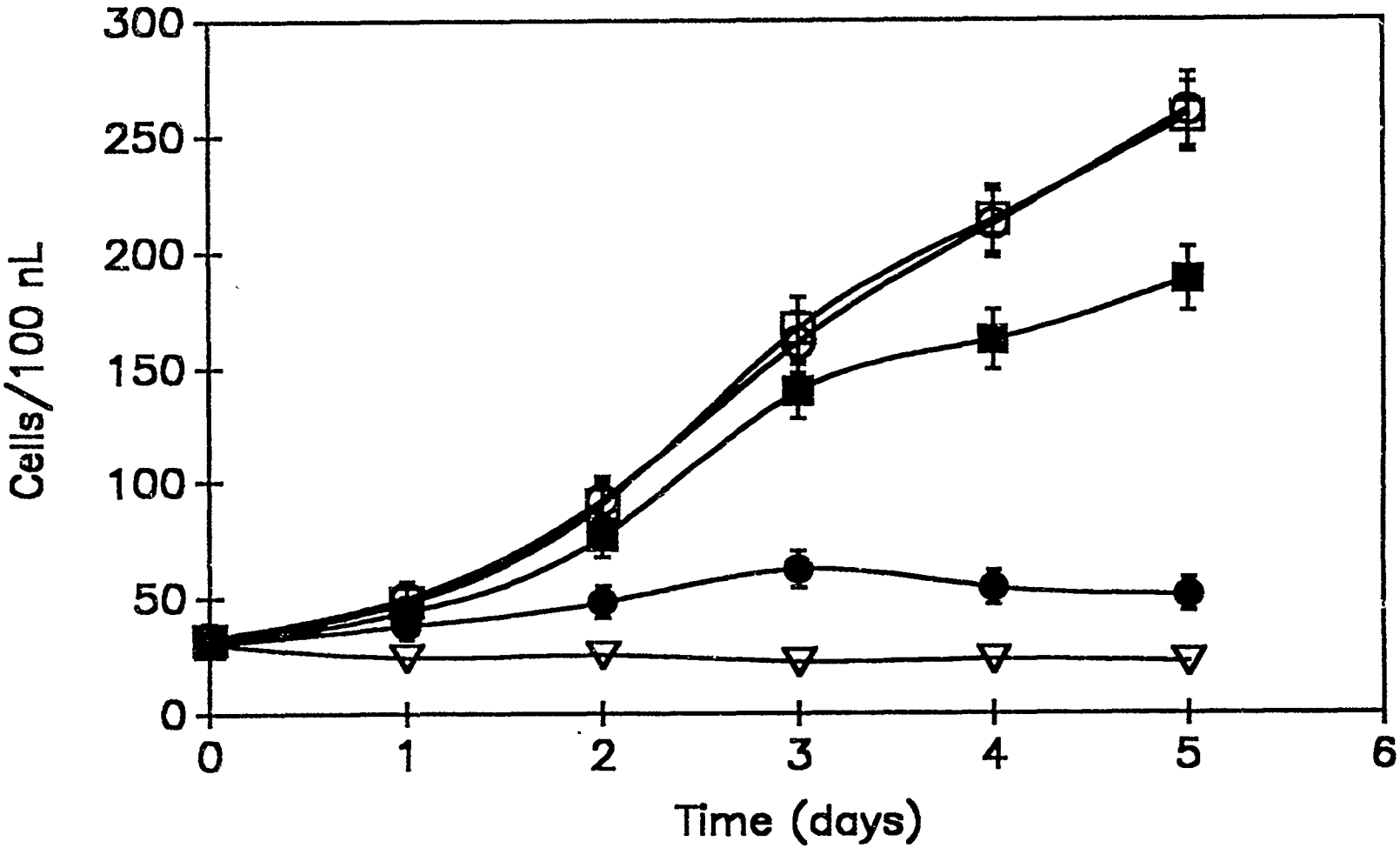

% Differentiated Granulocytes
100
80
60
40
20
0
−20
0
1
2
3
4
5
6
Time (days)

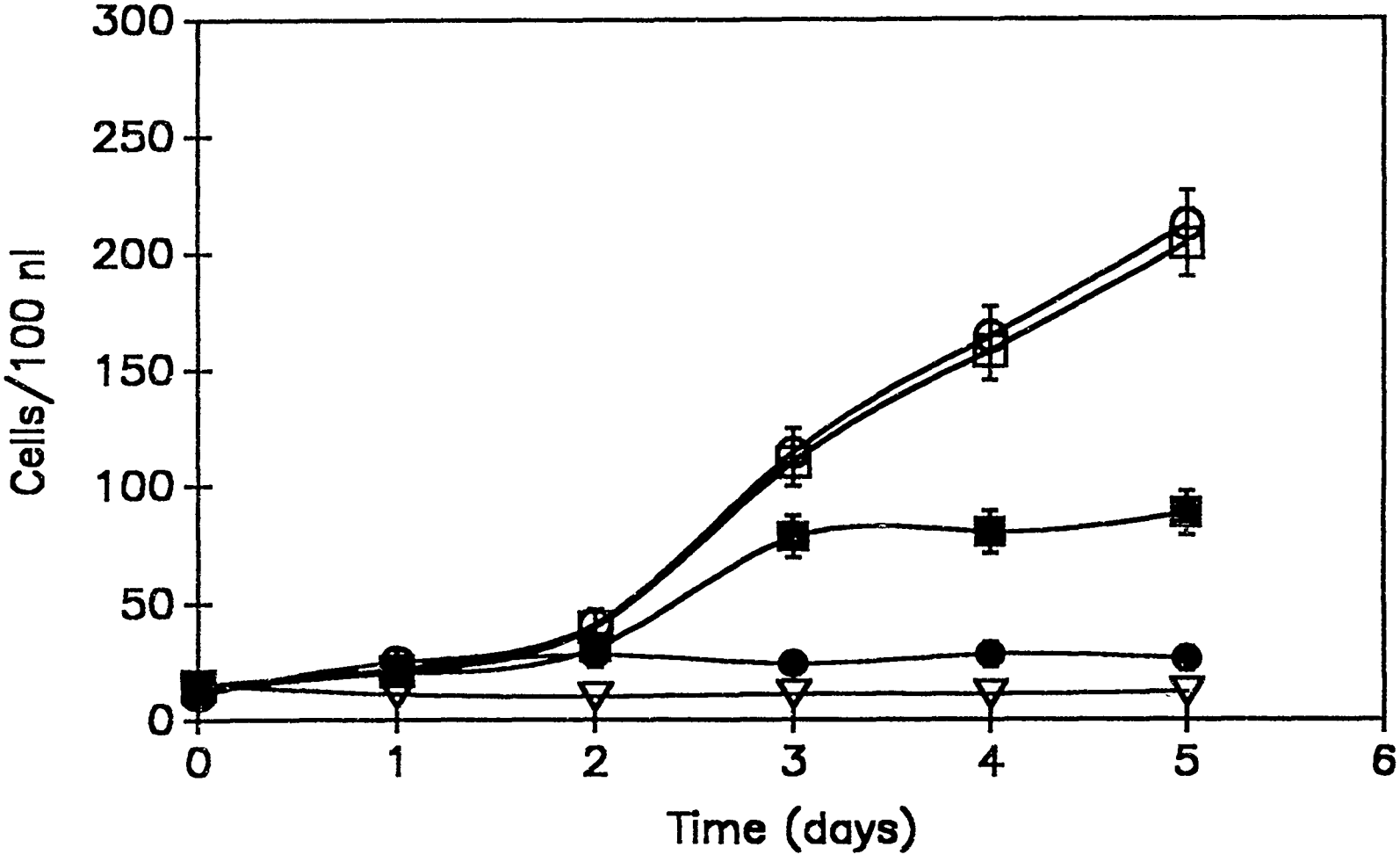

% Differentiated Granulocytes
100
80
60
40
20
0
−20
0
1
2
3
4
5
6
Time (days)

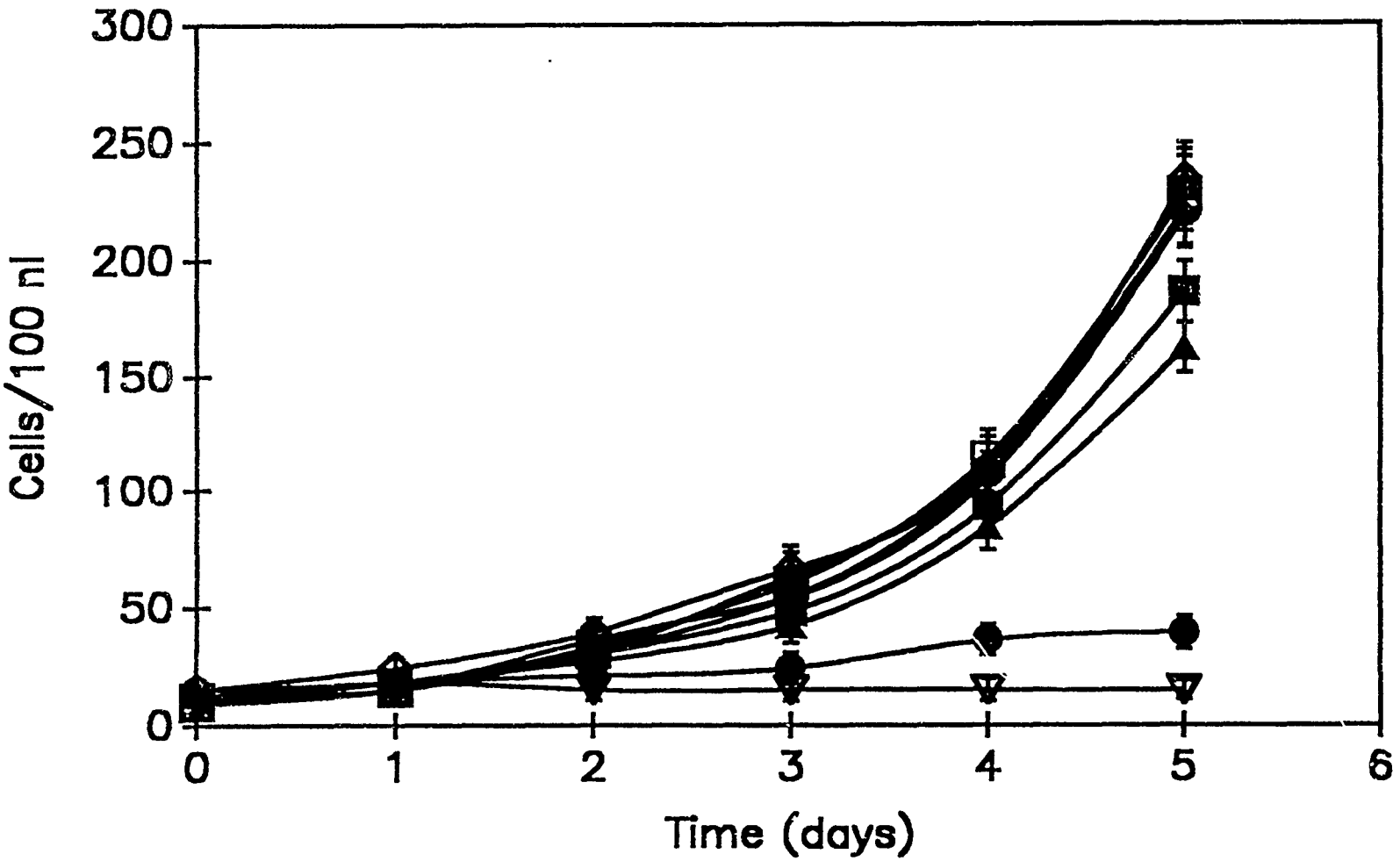

100
80
60
40
20
0
−20
% Sudan Black Positive
0
1
2
3
4
5
6
Time (days)

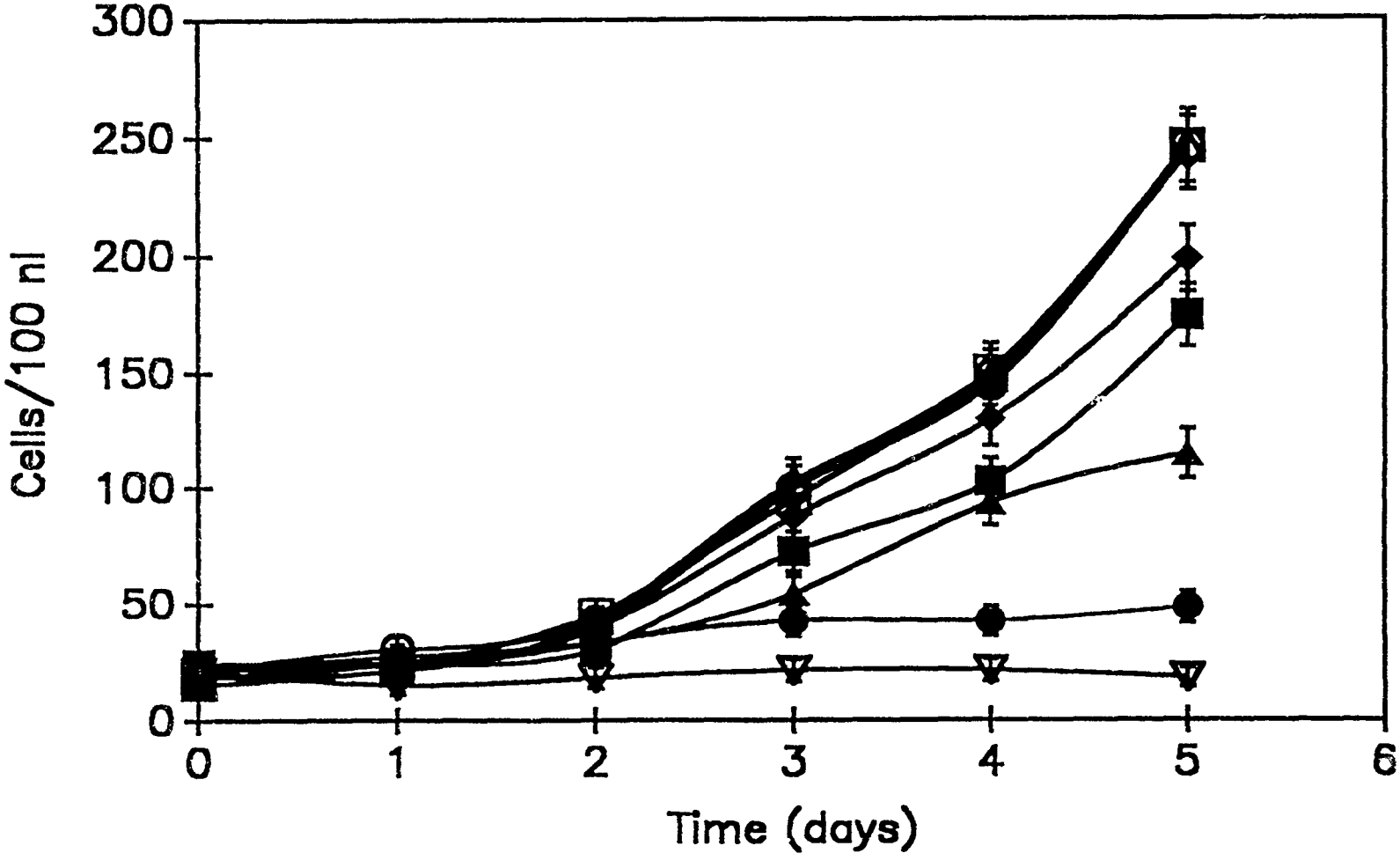

% NBT Reducing Cells
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)


% Naphthol AS–D Substrate Positive
100
80
60
40
20
0
–20
0
1
2
3
4
5
6
Time (days)


300
250
200
150
100
50
0
Cells/100 nL
0
1
2
3
4
5
6
Time (days)


300
250
200
150
100
50
0
Cells/100 nl
0
1
2
3
4
5
6
Time (days)


300
250
200
150
100
50
0
Cells/100 nl
0
1
2
3
4
5
6
Time (days)


300
250
200
150
100
50
0
Cells/100 nl
0
1
2
3
4
5
6
Time (days)

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Short Note

# α-Oligodeoxynucleotide stability in serum, subcellular extracts and culture media

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## Summary

Degradation of a synthetic α-oligodeoxynucleotide was studied in order to compare its survival with naturally occurring β-oligodeoxynucleotides in five systems used for antisense hybridization arrest experiments. In contrast to β-oligodeoxynucleotides, α-oligodeoxynucleotides were not detectably degraded over 24 h at 37 °C in HeLa cell postmitochondrial cytoplasmic extract or RPMI 1640 with 10% fetal bovine serum, and showed significant survival after 24 h at 37 °C in rabbit reticulocyte lysate, fetal bovine serum and human serum.

Key words. α-Oligodeoxynucleotide hydrolysis, Serum, human, Serum, fetal bovine, Reticulocyte lysate, rabbit; HeLa cell lysate; Deoxyribonuclease

## Introduction

Unmodified synthetic antisense oligodeoxynucleotides have been successfully used for sequence specific hybridization arrest of the expression of individual genes in vitro [1–6] and in cell cultures [7–11]. Unfortunately, oligodeoxynucleotides are rapidly degraded by serum enzymes [12], and degrade in cells with a half-life of 12 h or less [8,11].

Oligodeoxynucleoside methylphosphonates have been developed as nuclease resistant, uncharged oligodeoxynucleotide analogs, which have been successful as antisense inhibitors in cell culture [10,13–15]. Contrary to expectations, the greater

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longevity and lack of charge on oligodeoxynucleoside methylphosphonates have not resulted in significantly greater efficacy as antisense inhibitors than normal oligodeoxynucleotides. This is probably due to the asymmetry of the methylphosphonate moiety, yielding *R* and *S* diastereomers at each phosphodiester bond, where only the *S* form has the same structure as a normal oligodeoxynucleotide [16–18].

Oligodeoxynucleoside phosphorothioates, though ionic, display some nuclease resistance [19], and may be more efficacious than either oligodeoxynucleotides or oligodeoxynucleoside methylphosphonates as antisense inhibitors in cell culture [10]. They are also chiral, like the oligodeoxynucleoside methylphosphonates.

In an effort to avoid the problems of chirality, but maximize nuclease resistance, oligodeoxynucleotides have recently been synthesized with α-anomers of deoxynucleotides, rather than the natural β-anomers. These α-oligomers are achiral on phosphorus, are 1–2 orders of magnitude more resistant to several nucleases in vitro than are the normal β-oligomers, and hybridize strongly to β-oligomers and β-polymers in a parallel, rather than antiparallel manner [20–26].

The unnatural α-oligomers survive well in *Xenopus* oocytes, with a half-life of over 8 h, compared with only 10 min for β-oligomers [27]. The next question to be answered concerning α-oligodeoxynucleotides is their survival in other common experimental environments. We have made an attempt to answer this question by measuring the time course of labelled oligodeoxynucleotide degradation in rabbit reticulocyte lysate, HeLa cell postmitochondrial supernatant, RPMI 1640 medium with 10% fetal bovine serum, undiluted fetal bovine serum and adult human serum. In these systems, the α-oligomer half-lives observed were of the order of 24 h or more.

## Materials and Methods

### *α-Oligodeoxynucleotide synthesis*

The hexadecadeoxynucleotide α-[d(TAAAAGGGTGGGAATC)] was synthesized from the four 5′-*O*-dimethoxytrityl-α-2′-deoxynucleoside-3′-*O*-[(methyl)-*N*,*N*-(diisopropylamino)] phosphoramidites, coupled to 5′-OH-2-*N*-palmitoyl-α-2′-deoxyguanosine immobilized on controlled pore glass beads, using an Applied Biosystems 381A synthesizer, as described [28,29].

### *End labelling*

The hexadecadeoxynucleotide α-[d(TAAAAGGGTGGGAATC)] was 5′-end labelled with 5′-[γ-$^{35}$S]thioATP, 1300 Ci/mmol (du Pont/New England Nuclear), using $T_4$ polynucleotide kinase, incubated for 6 h at 37 °C and purified on a denaturing 20% polyacrylamide gel, as described [30].

### *Degradation reactions*

The labelled α-oligodeoxynucleotide was added to 25 μl aliquots of rabbit reticulocyte lysate (Promega Biotec), HeLa cell cytoplasmic extract, RPMI 1640

(Sigma) with 10% fetal bovine serum (Sigma), undiluted fetal bovine serum (Sigma) or undiluted adult human serum donated by one of us (T.A.B.). RPMI 1640 medium was developed at Roswell Park Memorial Institute, for the culture of human normal and neoplastic leukocytes [31,32]. The samples were incubated at 37 °C and 3 μl aliquots were removed at 1, 2, 4, 8 and 24 h. Reactions were terminated by the addition of each aliquot to 3 μl of 9 M urea, 10 mM EDTA, 0.05% xylene cyanol FF, 0.05% bromophenol blue.

*Analysis of products*

Each sample was analyzed by electrophoresis on denaturing 20% polyacrylamide gels as previously described [12], soaked in Fluorohance (RPI), dried onto Whatman 3MM paper, and fluorographed at −80 °C.

## Results

Three independent sets of degradation reactions and analyses were carried out, with similar results each time. Representative autoradiograms are shown in the

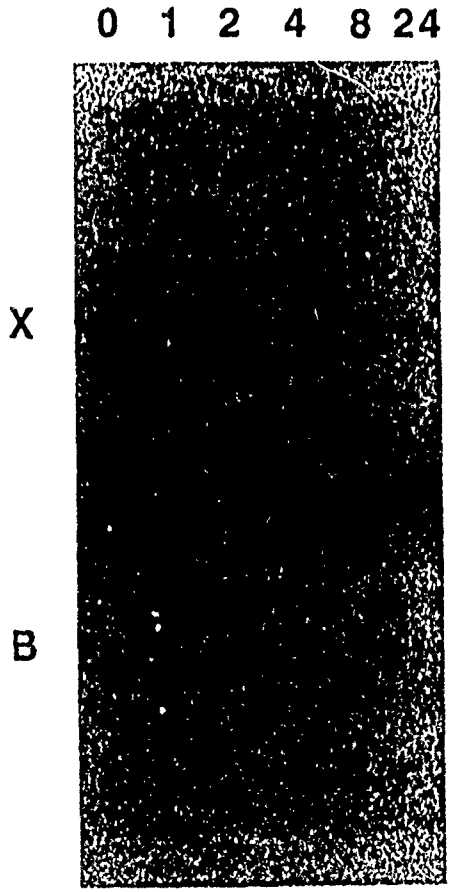


Fig. 1. Fluorogram of α-[5′-$^{35}$S]dTAAAAGGGTGGGAATC on denaturing 20% polyacrylamide gel after incubation in 25 μl rabbit reticulocyte lysate at 37 °C. Lane numbers correspond to aliquots of 3 μl which were removed at 1, 2, 4, 8 and 24 h. The first lane, 0, is a control not exposed to the extract, but containing the same number of cpm as any other sample, X and B show the mobilities of xylene cyanole FF and bromophenol blue, respectively.

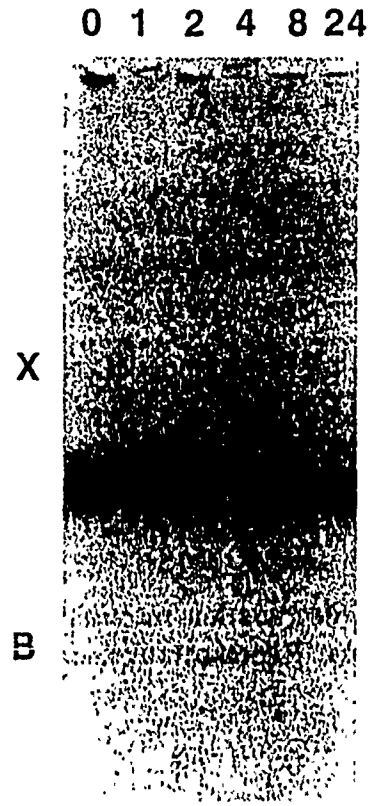


Fig. 2. Fluorogram of α-[5′-$^{35}$S]dTAAAAGGGAGGGAATC on denaturing 20% polyacrylamide gel after incubation in HeLa cell postmitochondrial extract, as in Fig. 1.

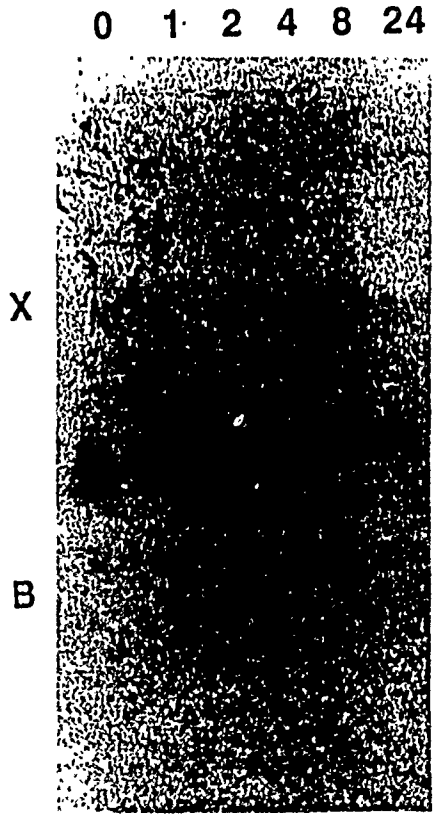


Fig. 3. Fluorogram of α-[5′-$^{35}$S]dTAAAAGGGAGGGAATC on denaturing 20% polyacrylamide gel

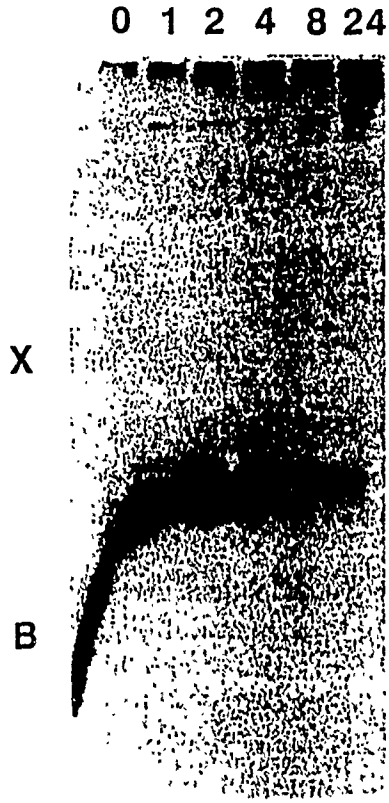


Fig. 4. Fluorogram of α-[5′-$^{35}$S]dTAAAAGGGAGGGAATC on denaturing 20% polyacrylamide gel after incubation in undiluted fetal bovine serum, as in Fig. 1.

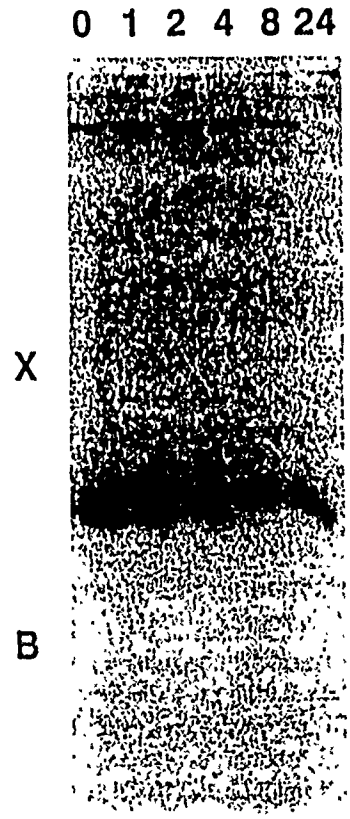


Fig. 5. Fluorogram of α-[5′-$^{35}$S]dTAAAAGGGAGGGAATC on denaturing 20% polyacrylamide gel after incubation in undiluted adult human serum, as in Fig. 1.

figures below. No significant α-oligodeoxynucleotide degradation was detected in rabbit reticulocyte lysate over the first 8 h, but by 24 h, a decrease of about one third was seen (Fig. 1).

In HeLa cell postmitochondrial cytoplasmic extracts, however, no degradation was seen at any time point (Fig. 2). Similarly, in RPMI 1640 with 10% fetal bovine serum, no significant degradation was detected at any time point (Fig. 3).

In undiluted fetal bovine serum, on the other hand, no degradation was apparent over the first 8 h, but between 8 and 24 h a decrease of about one half was seen (Fig. 4).

Undiluted human serum showed a slow steady decrease after 2 h, having decreased by over one half at 24 h (Fig. 5).

## Discussion

Degradation of β-oligodeoxynucleotides was studied earlier [12] in four biological systems in order to assess the importance of oligodeoxynucleotide hydrolysis during hybridization arrest experiments. 5′-[γ-$^{32}$P]Oligodeoxynucleotides were tested as above in rabbit reticulocyte lysate, HeLa cell postmitochondrial cytoplasmic extract, DMEM with 5% fetal bovine serum or undiluted bovine serum. No degradation was detected over 90 min at 37 °C in rabbit reticulocyte lysate or DMEM plus 5% fetal bovine serum, while in HeLa cell cytoplasmic extract, degradation to families of shorter oligodeoxynucleotides showed a half-life of about 30 min. Most importantly in undiluted fetal bovine serum, degradation was complete within the first 15 min. The very rapid degradation seen in fetal bovine serum confirmed the expectation that unprotected oligodeoxynucleotides could not be used in a whole animal experiment through the bloodstream route.

In the studies of α-oligodeoxynucleotide degradation described above, it is clear that degradation is quite slow. Even in undiluted fetal bovine or adult human serum, a significant fraction of the labelled α-oligodeoxynucleotide was still intact after 24 h. Furthermore, the absence of a ladder of shorter oligodeoxynucleotides implies that disappearance of labelled α-oligodeoxynucleotides from the gel was not due to endonuclease or 3′-exonuclease activity, but rather to 5′-phosphatase or 5′-exonuclease activity. Hence, α-oligodeoxynucleotides represent a plausible method for attempting antisense oligodeoxynucleotide hybrid arrest in cultured cells and whole animal systems, with reasonable expectation of slow turnover.

## Simplified description of the method and its applications

Antisense oligodeoxynucleotide hybridization arrest of mRNA translation depends on minimal degradation of the oligodeoxynucleotide during the experiment. This work shows that one may carry out a hybridization arrest experiment with an unprotected α-oligodeoxynucleotide for up to 24 h in rabbit reticulocyte lysate, HeLa cell postmitochondrial lysate, RPMI 1640 with 10% fetal bovine serum, undiluted fetal bovine serum or adult human serum. Hence, whole animal experiments should be feasible by intravenous administration.

## Acknowledgements

We thank Dr. Lee A. Weber for a sample of HeLa cell postmitochondrial supernatant. This work was supported by grants from the US National Institutes of Health, CA 42960 and RR-07121, the US Army Medical Research and Development Command, DAMD17-86-G-6037, the American Foundation for AIDS Research, 00004, the Leukemia Society of America, and the American Cancer Society Florida Division, to E.W., and from the Association pour la Recherche sur le Cancer de France, to J.-L. I.

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# FMOC- MEDIATED SOLID PHASE ASSEMBLY OF HIV TAT PROTEIN

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## Introduction

The human immunodeficiency virus (HIV) encodes for several regulatory proteins which are essential for expression. The Tat protein, directly or indirectly, increases the utilization of mRNA. In human cells Tat causes an increase in the level of mRNA by approximately 10 times, whereas the amount of protein produced increases 500 fold. Tat is of relatively small size (86 residues), but its unusual composition and complex sequence pose exception synthetic problems. These include the presence of a strongly basic Arg rich region which might bind nucleic acids, the presence of many Gln residues, and also of 7 Cys residues. All Cys residues exist in free SH forms coordinated to 4 zinc atoms in a dimer. The synthesis of even uncomplicated proteins remains fraught with uncertainties. Almost all examples have employed the classical Merrifield method of synthesis, although the harsh acid deprotection is damaging in sensitive cases. One aim of this work was to test improvements to Fmoc protocols developed in the Biosearch laboratories. Other aims were to obtain sufficient pure material to analyze the structure and function of Tat and of partially protected forms and fragments.

## Results

Polystyrene was selected for the support rather than encapsulated polydimethylacrylamide. The efficient (1) and generally useful (2) BOP + HOBt coupling method was adopted. The synthesis was performed on a MilliGen/Biosearch Model 9600 using protection and coupling times as shown in the table below.

HIV-*tat* Protein:

Sequence, Protection and Coupling Information

| Residue | Met | Glu | Pro | Val | Asp | Pro | Arg | Leu | Glu | Pro | Trp | Lys | His |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Protection | - | OBut | | - | OBut | | Mtr | - | OBut | - | - | Boc | Fmoc |
| Coupling Time (hrs) | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

| Residue | Pro | Gly | Ser | Gln | Pro | Lys | Thr | Ala | Cys | Thr | Asn | Cys | Tyr |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
| Protection | | | But | Tmob | | Boc | But | - | Trt | But | - | Trt | But |
| Coupling Time (hrs) ▲ | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

| Residue | Cys | Lys | Lys | Cys | Cys | Phe | His | Cys | Gln | Val | Cys | Phe | Ile |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
| Protection | Acm | Boc | Boc | Acm | Acm | | Fmoc | Trt | Tmob | - | Trt | - | - |
| Coupling Time (hrs) | 2 | 2 | 2 | 2 | 2 | 2 | 2 ▲ | 2 | 2 | 2 | 2 | 2 | 2 |

| Residue | Thr | Lys | Ala | Leu | Gly | Ile | Ser | Tyr | Gly | Arg | Lys | Lys | Arg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 |
| Protection | But | Boc | - | | | | But | But | - | Mtr | Boc | Boc | Mtr |
| Coupling Time (hrs) | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 2 ▲ | 2 |

| Residue | Arg | Gln | Arg | Arg | Arg | Pro | Pro | Gln | Gly | Ser | Gln | Thr | His |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 |
| Protection | Mtr | Tmob | Mtr | Mtr | Mtr | | | Tmob | - | But | Tmob | But | Fmoc |
| Coupling Time (hrs) | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |

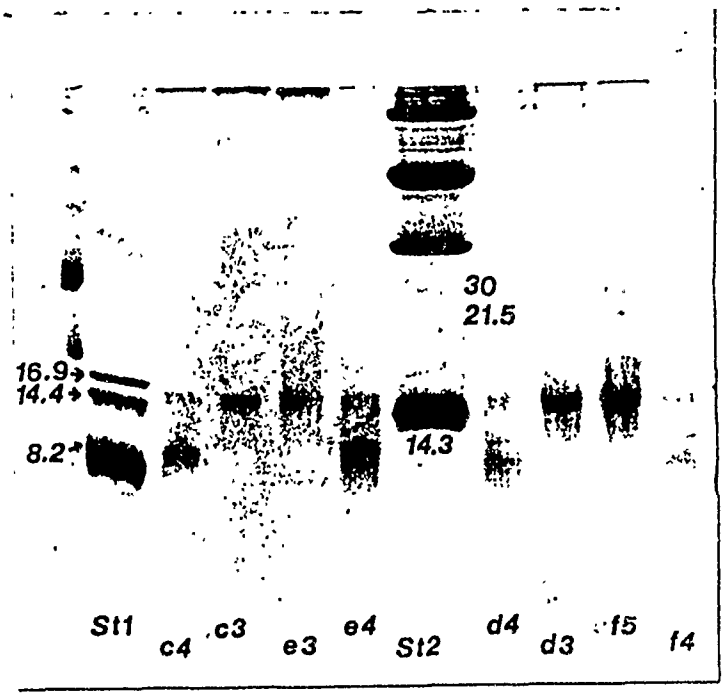
| Residue | Gln | Val | Ser | Leu | Ser | Lys | Gln | Pro | Thr | Ser | Gln | Ser | Arg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 |
| Protection | Tmob | | But | | But | Boc | Tmob | - | But | But | Tmob | But | Mtr |
| Coupling Time (hrs) | 2 | 1 ▲ | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 |

| Residue | Gly | Asp | Pro | Thr | Gly | Pro | Lys | Glu |
|---|---|---|---|---|---|---|---|---|
| No. | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 |
| Protection | | OBut | | But | | | Boc | OBut |
| Coupling Time (hrs) | 1 | 1 | 1 | 1 | 1 | 1 | 2 | |

Samples, arrowed, and the final product were treated with Reagent R (TFA/Thioanisole/ethane dithiol/anisole; 90:5:3:2, 8 hours) which cleanly removed Mtr protection. The products were assessed by HPLC, AAA and sequencing. All peptides gave single main peaks on HPLC after DTT reduction, and sequenced correctly. No preview resulting from incomplete coupling was detected. The figure on the following page shows the polyacrylamide gel electrophoresis of fully reduced and tris Acm forms of materials from G50-50 Sephadex chromatography.

Figure:

SDS- Gel Electrophoresis of 86-mer fractions




3 or 4 designates void volume or subsequent fraction respectively; d & f are tris (Acm) derivatives, c & e are after $Hg(OAc)_2$ treatment; c & d are cleaved with Reagent R for 8 hours, f & e for 16 hours, standards were: St1 is Sigma MWSD517, St2 Amersham "Rainbow Markers".

## Conclusions

The results demonstrate a highly efficient assembly of one of the most complex series of peptides yet prepared by Fmoc-mediated solid phase synthesis. No data is available on biological activity as yet.

## Acknowledgement

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Classification: Biochemistry

# Walking along human c-*myc* mRNA with antisense oligodeoxynucleotides: maximum efficacy at the 5' cap and initiation codon regions

Key words: oncogenes/hybrid arrest/promyelocytic leukemia/HL-60 cells/RNA structure

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Abbreviations: p65, the 65 kD c-*myc* protein; nt, nucleotide.

**ABSTRACT** The nature of antisense oligodeoxynucleotide inhibition of translation and its relation to the predicted secondary structure of human c-*myc* oncogene mRNA were examined. A series of different antisense pentadecamers complementary to predicted loops, bulges and helices between the cap and initiation codon regions of c-*myc* mRNA were synthesized. HL-60 cells in culture were treated for 24 hr with 1 - 10 $\mu$M of each oligomer, plus controls. The levels of c-*myc* p65 antigen were then analyzed by radioimmunoprecipitation and laser densitometry. The efficacy of the cap antisense sequence in reducing p65 expression was roughly twice that of the original [Wickstrom, E. L., Bacon, T. A., Gonzalez, A., Freeman, D. L., Lyman, G. H. and Wickstrom, E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 1028-1032] initiation codon antisense sequence. However, the other target sequences downstream of the cap, and up to the initiation codon, were much less effective. At the initiation codon target, a dodecamer was about half as effective as the original pentadecamer, as was a pentadecamer with two mismatches. Similarly, an octadecamer was about twice as effective. However, these differences are much less dramatic than a simple thermodynamic model would have

predicted. The observation of variation in antisense efficacy as a function of target location in c-*myc* mRNA may represent a combination of the effects of both RNase H attack, at all targets, and genuine hybrid arrest of translation, at the sensitive cap and initiation codon sites. Alternatively, the latter two targets might be those which are the most exposed in the tertiary structure of c-*myc* mRNA.

The ability to turn off individual genes at will in growing cells provides a powerful tool for elucidating the role of a particular gene, and for therapeutic intervention when that gene is overexpressed. In principle, one needs to identify a unique target sequence in the gene of interest, and prepare a complementary oligonucleotide against the target sequence, in order to disrupt translation (1). This approach, which is called antisense inhibition (2), may utilize either RNA (3) or DNA (4). Antisense oligodeoxynucleotides have been utilized against a wide variety of target genes, in viral, bacterial, plant, and animal systems, both in cell-free extracts and in whole cells (5 (rev.)).

Cancerous cells display overexpression or mutant expression of one or more of the genes normally used in cell proliferation. Such genes are

called proto-oncogenes (6). The proto-oncogene c-*myc*, an evolutionarily conserved gene found in all vertebrates, has been found to be overexpressed in a wide variety of human leukemias and solid tumors (7). The c-*myc* gene expresses a nuclear protein with an electrophoretic apparent molecular mass of 65 kD (p65) (8,9), and overexpression of p65 promotes replication of SV40 DNA (10). Inhibition of c-*myc* p65 expression by an antisense oligodeoxynucleotide targeted against a predicted loop containing the initiation codon of the human c-*myc* mRNA was found to inhibit mitogen-stimulated human peripheral blood lymphocytes from entering S phase (11), and was observed to inhibit HL-60 cells from proliferating (12), in a sequence-specific, dose-dependent manner. Hence, it appears likely that the c-*myc* gene product plays some direct or indirect role in replication. No characteristic DNA binding sequence has been found for p65, and its amino acid sequence places it in the category of leucine zipper proteins, which are theorized to regulate gene expression by interactions with other similar proteins along their leucine zipper $\alpha$-helical backbones (13).

Furthermore, overexpression of p65 usually correlates with inability of cells to differentiate (14,15). In contrast, induction of HL-60 cell differentiation with $Me_2SO$ coincides with a decline in c-*myc* mRNA (16) and the ability of the HL-60 cells to form colonies in semisolid medium (17). It now appears that c-*myc* p65 derepresses negatively regulated proliferative genes at the transcriptional level (18). Like $Me_2SO$, the anti-c-*myc* initiation region oligomer was found to elicit a sequence-specific increase in HL-60 cell differentiation along the granulocytic line, and inhibition of colony formation in semisolid medium (19). Daily addition of anti-c-*myc* oligomer for five days was fully as effective as 1% $Me_2SO$ (Bacon, et al., unpublished results). On the other hand, c-*myc* antisense RNA was reported to induce HL-60 cell differentiation along the monocytic line (20).

The mechanism of antisense oligodeoxynucleotide inhibition was first assumed to be hybridization arrest of mRNA translation by ribosomes (4), but also clearly depends on RNase H attack on the RNA/DNA hybrid formed between the mRNA and the antisense oligodeoxynucleotide (21). Our choice of the c-*myc* mRNA initiation codon region as a target was

based on calculation of a possible secondary structure (Fig. 1), in the absence of experimental evidence. Calculations of mRNA secondary structures often place the initiation codon in a single stranded region following a highly basepaired 5' untranslated leader (25). In an effort to test the validity of the secondary structure prediction, a series of different antisense pentadecamers complementary to predicted loops, bulges and helices between the cap and initiation codon regions of c-*myc* mRNA were synthesized, and their efficacy as antisense inhibitors was measured. The dependence of antisense inhibition at the initiation codon target on oligomer length was also examined.

## MATERIALS AND METHODS

**Cell Culture.** HL-60 cells were grown and maintained in log phase with greater than 90% viability in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) at 37°C in a 5% $CO_2$ atmosphere saturated with water. All tissue culture media contained $10^5$ units of penicillin, 0.1 g streptomycin and 0.5 g gentamicin per liter. Cell titers and viability were determined by trypan blue (Gibco) dye exclusion.

**Cell Treatment**. HL-60 cells were sedimented, decanted, and resuspended in fresh culture medium to a concentration of 2 x $10^5$ cells/ml, and then incubated for 24 hrs in the wells of culture plates in order to re-establish logarithmic growth before addition of modulators. Antisense oligodeoxynucleotides (Table 1) were synthesized on a Biosearch 8750 DNA synthesizer, purified as before (12) on an ISCO liquid chromatograph, and added directly to cell suspensions at concentrations detailed in the text.

**Radioimmunoprecipation of c-myc p65 Protein**. Samples of 2 x $10^6$ cells from the HL-60 culturé were sedimented, decanted, washed in PBS, and resuspended in 1 ml of cysteine/methionine-free RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, L-cysteine, (1066 Ci/mmol; 1 Ci = 37 GBq; du Pont/New England Nuclear) at 300 $\mu$Ci/ml and L-methionine, (1198 Ci/mmol; du Pont/New England Nuclear) at 300 $\mu$Ci/ml. Each sample was grown for an additional 1.5 hr, after which the cells were sedimented, washed, lysed, immunoprecipitated, electrophoresed, fluorographed, and quantitated by densitometry as described (12).

## RESULTS

**Prediction of antisense targets.** Calculation of one possible secondary structure for the entire 2121-nt human c-*myc* mRNA from K562 human erythroleukemia cells (12) (Fig. 1) placed the initiation codon in a large bulge loop 559 nt downstream from the cap. This predicted loop was chosen as the initial target for antisense inhibition, on the assumption that those nucleotides might be readily available for hybridization arrest. The efficacy of c-*myc* inhibition which we found by utilizing this target (11,12,19) was consistent with its predicted accessibility, but is hardly a test of the secondary structure model. Hence, a series of antisense oligodeoxynucleotides was prepared against a series of predicted stems, loops, and bulges from the cap to the initiation codon region, including a predicted hairpin just downstream of the AUG (Table 1). If it is assumed that antisense inhibition depends on the accessibility of different portions of mRNA to their complementary oligomers, then one expects that the effectiveness of each of the sequences in Table 1 should correlate with the extent of secondary structure at each mRNA target.

Similarly, the effectiveness of antisense inhibition at each target in c-*myc* mRNA should increase with the length of the antisense oligomer, in proportion to the increased free energy of binding. If one literally assumed that an antisense 12-mer, 15-mer, and 18-mer directed against the predicted initiation codon loop could bind freely with all complementary residues in the mRNA target, then the association constant of a 15-mer would be orders of magnitude greater than that of a 12-mer, and the binding of an 18-mer would be ( rders of magnitude greater than that of the 15-mer. In order to test this simple model, antisense oligomers were synthesized against the initiation codon and the next three, four, and five codons (Table 1). The effects of mismatches were examined in one pentadecamer analog of the initation codon antisense sequence which contained two nonadjacent mismatches.

**Efficacy at different sites.** The efficacy of each oligomer was measured by radioimmunoprecipitation of c-*myc* p65 antigen from untreated HL-60 cells and cells treated with 10 $\mu$M of each oligomer for 24 hr (Fig. 2). Quantitation of p65 bands appears in Fig. 3. The degree of variation in this method may be seen by comparing control lanes B and U,

as well as sequence control lanes D and E. The 5' cap sequence (F), which appears in the predicted structure as a weak stem and bulge region, was more than twice as sensitive a target as the original initiation codon sequence (O), which occurs in an even weaker bulge and stem area in Fig. 1. Even the oligomer directed against nt 9-23 (G), slightly downstream from the cap, was more effective than the initiation codon oligomer, despite the prediction that sequence G was included in a strong stem.

Sequence H, directed against a predicted hairpin loop and bulge beginning 127 nt downstream from the cap, was less effective than our standard sequence O, as were targets I, supposedly a weak helical region, K, a putative hairpin loop, and M, which began in a helical region upstream of the initiation AUG, and overlapped sequence O by 7 nt. The efficacy of sequences J, calculated to be a tight hairpin, and L, predicted to occur in a weak helix, were comparable to the initiation codon sequence O. Sequence S, just downstream of the initiation codon sequence, showed almost no inhibition.

Upon varying the length of the initiation codon probes, it was found that sequence N, which was only 12 nt, was half as effective as the 15-mer

O, while the 18-mer R was about twice as effective. The analog of sequence O containing two separated mismatches, P, was less effective than O, but more so than the 12-mer.

**Concentration dependence of antisense inhibition.** The strong efficacy at the 5' end, and the length dependence of inhibition at the initiation codon target, were studied as a function of oligomer concentration, in order to test the validity of the observations at 10 $\mu$M (Fig. 4). Despite significant variability, it was apparent that antisense inhibition was dose dependent, and that the relative efficacies at 10 $\mu$M (Fig. 3) were consistent with those seen in the concentration ramps.

## DISCUSSION

The pattern of antisense inhibition as a function of target sequence described above for human c-*myc* mRNA does not correlate with the particular secondary structure of the message predicted in Fig. 1. The most sensitive sites were the 5' cap and the initiation codon regions. Similar results have been found for antisense oligodeoxynucleotide inhibition of rabbit globin mRNA translation in cell-free extracts (27,28).

On the other hand, antisense inhibition of human immunodeficiency virus mRNA translation in infected cells revealed that the most efficacious targets in the viral system were the 3' polyadenylation signal, 5' leader sequences at nt 54-73 and 162-181, and the splice acceptor site at nt 5349-5368, rather than the cap or initiation codon, which were a little less sensitive (29).

To the extent that antisense inhibition depends on genuine hybrid arrest of ribosomal translation of an mRNA, it is logical that those sites in a message which are recognized by initiation factors would be the most sensitive. To the extent that antisense inhibition depends on RNase H hydrolysis of antisense oligodeoxynucleotide/mRNA hybrids, the sensitivity of each site in a message should correlate with its accessibility, as determined by its secondary and tertiary structure. From the observations presented above for c-*myc* mRNA in whole HL-60 cells, RNase H activity may be a secondary effect in this system relative to hybrid arrest of initiation. On the other hand, it is also possible that the most sensitive sites are those which are most exposed in the tertiary structure of c-*myc*

mRNA, which would allow preferential binding to antisense oligomers, and resulting hydrolysis by RNase H.

Independent of mechanism, the much greater sensitivity of the cap and initiation codon sequences to antisense inhibition is also at odds with the scanning model for ribosomal translation of eukaryotic messages (30). Human c-*myc* mRNA has a 558 nt untranslated 5' leader, which contains no upstream AUGs (22), but also initiates at a CUG 14 codons upstream of the AUG initiation codon (31). In principle, any DNA/RNA hybrid from the cap to the genuine initiation codon should inhibit scanning similarly, but this was not observed for c-*myc* mRNA. Indeed, most oncogene mRNAs whose sequences are known possess long 5' untranslated leaders with upstream AUGs, yet they are efficient messages (25). Perhaps oncogene mRNAs share with some viral mRNAs the ability to initiate translation directly at internal AUGs without scanning (32,33).

Eukaryotic initiation factors eIF4F and eIF4B are essential for mRNA association with 40S ribosomal subunits (34). It has been observed that wheat germ initiation factor eIF4B binds to the initiation codon region of uncapped satellite tobacco necrosis virus RNA (35), and that rabbit

reticulocyte eIF4B binds specifically to AUG (36). Both eIF4F, which includes eIF4A, eIF4E, and a 220 kD subunit, and eIF4B may be crosslinked to the oxidized 5' cap region of mRNA, if ATP is present (34). These observations allow for the possibility that the 5' cap and the AUG used for initiation are located close to each other in the tertiary structure of human c-*myc* mRNA, and interact either sequentially (25) or simultaneously with initiation factors to effect initiation.

The modest dependence of antisense effectiveness on oligomer length at the initiation codon target, and the residual efficacy of the pentadecamer with two mismatches, imply that none of the oligomers employed against the initiation codon region hybridize completely to the message. This is consistent with the prediction in Fig. 1 that the AUG is exposed in a bulge, rather than a large loop, as predicted for a 400 nt portion of c-*myc* mRNA centered on the initiation codon (12). If antisense inhibition is most effective at cap and initiation codon sequences, and a more modest phenomenon in untranslated leaders and coding regions, then even in a human system a 12-mer may display sufficient statistical uniqueness, among the available targets, for gene-specific inhibition.

The dependence of antisense inhibition on mRNA secondary and tertiary structure, and the relevance of cap and initiation codon sensitivity to the mechanism of translational initiation, should be tested in more detail. Additional targets should be probed, such as the upstream initiator CUG, splice junctions in the original transcript, more sites in the coding region, and in the 3' tail. At the most sensitive sites, concentration dependence should be studied for each oligomer size over a broader range, in order to determine the optimum sequence and length for maximum efficacy and specificity. The independence of the cap and AUG sites should be examined by assessing the effectiveness of combinations of their respective antisense oligodeoxynucleotides. Finally, the separation of the cap and initiation codon within the mRNA tertiary structure should be tested by preparing oligomers containing both antisense sequences in a single molecule, separated by variable length deoxynucleotide molecular rulers, and measuring their efficacy.

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Table 1. Antisense Oligodeoxynucleotide Target Sequences

| Target Label | Target Location | Antisense Sequence |
|---|---|---|
| D | VSV-M | 5'-d(TTG GGA TAA CAC TTA)-3' |
| E | -45 to -31 | 5'-d(CTC GCA TTA TAA AGG)-3' |
| F | 1 to 14 | 5'-d(GCA CAG CTC GGG GGT)-3' |
| G | 9 to 23 | 5'-d(CCG GCG GTG GCG GCC)-3' |
| H | 127 to 141 | 5'-d(GCC CCG AAA ACC GGC)-3' |
| I | 207 to 221 | 5'-d(CGC CCG GCT CTT CCA)-3' |
| J | 277 to 291 | 5'-d(TGG GCC AGA GGC GAA)-3' |
| K | 382 to 396 | 5'-d(GTG TTG TAA GTT CCA)-3' |
| L | 536 to 550 | 5'-d(GAG GCT GCT GGT TTT)-3' |
| M | 551 to 565 | 5'-d(GGG GCA TCG TCG CGG)-3' |
| N | 559 to 570 | 5'-d(GTT GAG GGG CAT)-3' |
| O | 559 to 573 | 5'-d(AAC GTT GAG GGG CAT)-3' |
| P | 559 to 573 | 5'-d(AAC GTT GtG GaG CAT)-3' |
| Q | 559 to 573 | 5'-d(CTG AAG TGG CAT GAG)-3' |
| R | 559 to 567 | 5'-d(GCT AAC GTT GAG GGG CAT)-3' |
| S | 574 to 588 | 5'-d(CCT GTT GGT GAA GCT)-3' |

Legend to Table 1

Antisense oligodeoxynucleotide sequences targeted against predicted stems, loops and bulges in the calculated secondary structure of the human c-*myc* mRNA (Fig. 1). Labels correspond to targets in Fig. 1 and lanes in Figs. 3 and 4. Sequences are numbered according to the c-*myc* mRNA sequence of Watt, et al. (22). Negative control sequences included nt 17-31 of VSV matrix protein mRNA (D), a sequence in the gene (26) upstream of the transcription start site (E), and a scrambled version (Q) (11) of the initiation codon sequence (O). Lower case letters represent mismatches between sequence P and the initiation codon region target, 559-573.

Fig. 1. Predicted secondary structure of the entire 2121-nt human c-*myc* mRNA from K562 cells (22) calculated with the RNAFLD (23) using free energies of base pairing at 37°C (24). Target locations are indicated with capital letters.

Fig. 2. Expression of c-*myc* p65 protein in treated and untreated HL-60 cells, measured by radioimmunoprecipitation and fluorography. Antisense oligodeoxynucleotides were added directly to the suspensions at a final concentration of 10 $\mu$M for 12 hr. Lane A, molecular weight markers; lane B, no oligomer, anti-p65 antibody; lane C, no oligomer, rabbit IgG; lanes D-S, oligomers from Table 1, anti-p65 antibody; lane T, no oligomer, rabbit IgG; lane U, no oligomer, anti-p65 antibody; lane V, molecular weight markers.

Fig. 3. Relative intensities of c-*myc* p65 protein expression from Fig. 2. Each band was scanned at three different points with an LKB 2220 laser densitometer and the intensities were averaged. The entire scanning routine was repeated, and error bars represent the standard deviation of the six scans. B and U, no oligomer, anti-p65

antibody; C and T, no oligomer, rabbit IgG; D-S refer to oligomers in Table 1, with anti-p65 antibody.

Fig. 4. Relative intensities of c-*myc* p65 protein expression in untreated cells (wide right crosshatch) or HL60 cells treated with 1 $\mu$M (narrow right and left crosshatch), 2.5 $\mu$M (narrow right crosshatch), 5 $\mu$M (blank box) or 10 $\mu$M (solid box) of each antisense oligodeoxynucleotide. Antigens were radioimmunoprecipitated, fluorographed and scanned as in Figs. 2 and 3. B, no oligomer, anti-p65 antibody; C, no oligomer, rabbit IgG; F, G, N, O, and R refer to oligomers in Table 1 and lanes in Fig. 2, with anti-p65 antibody.

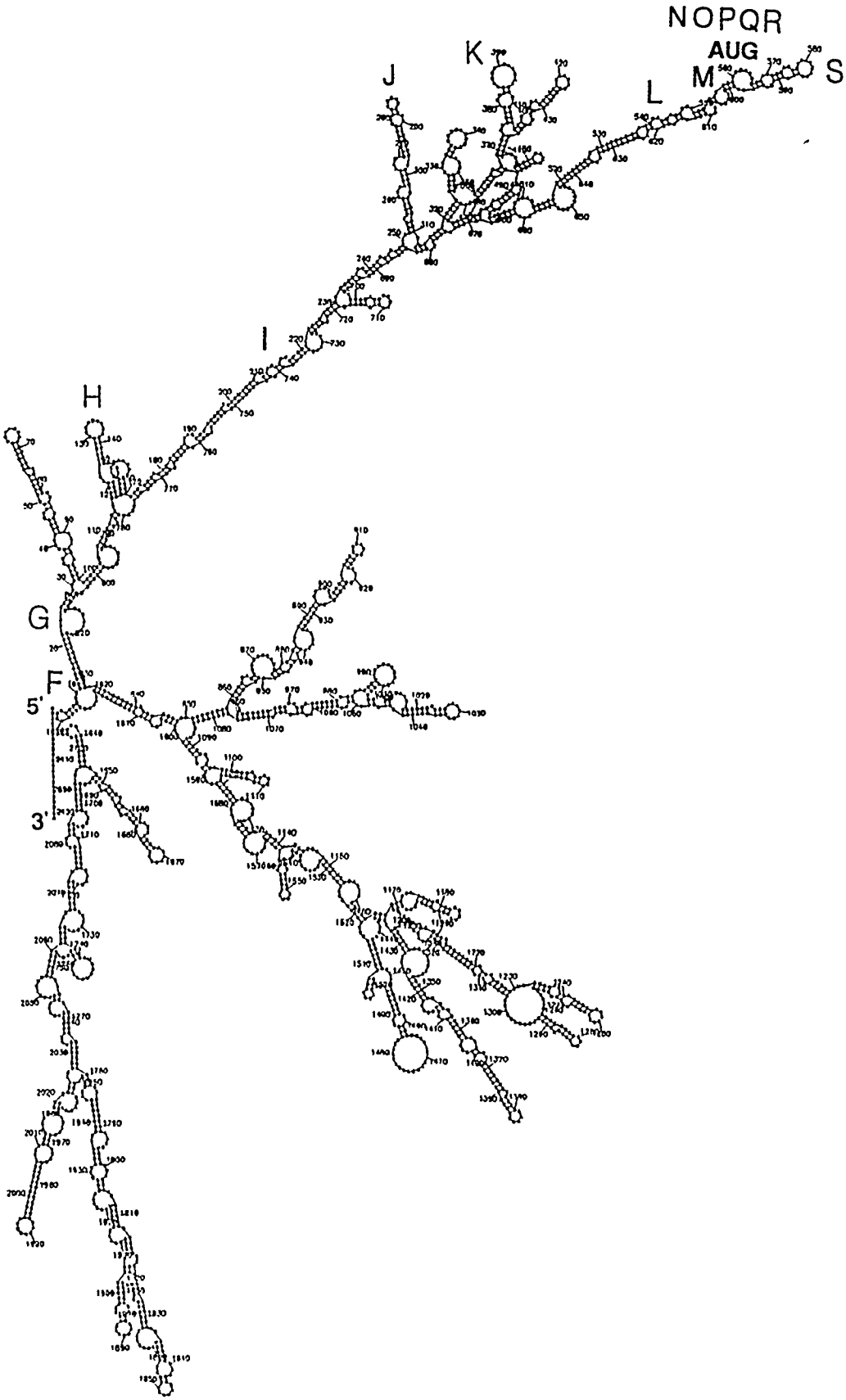



Fig. 1

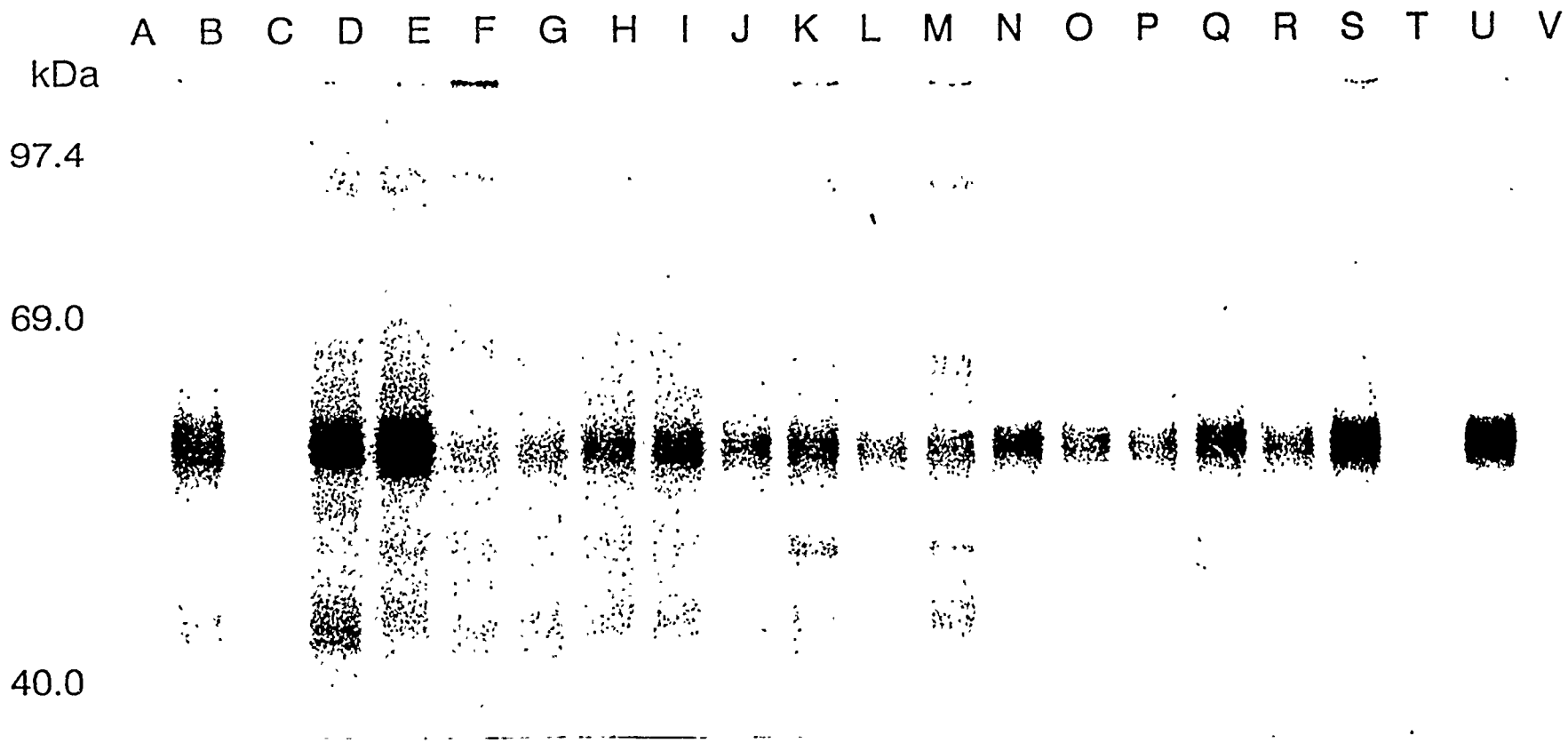
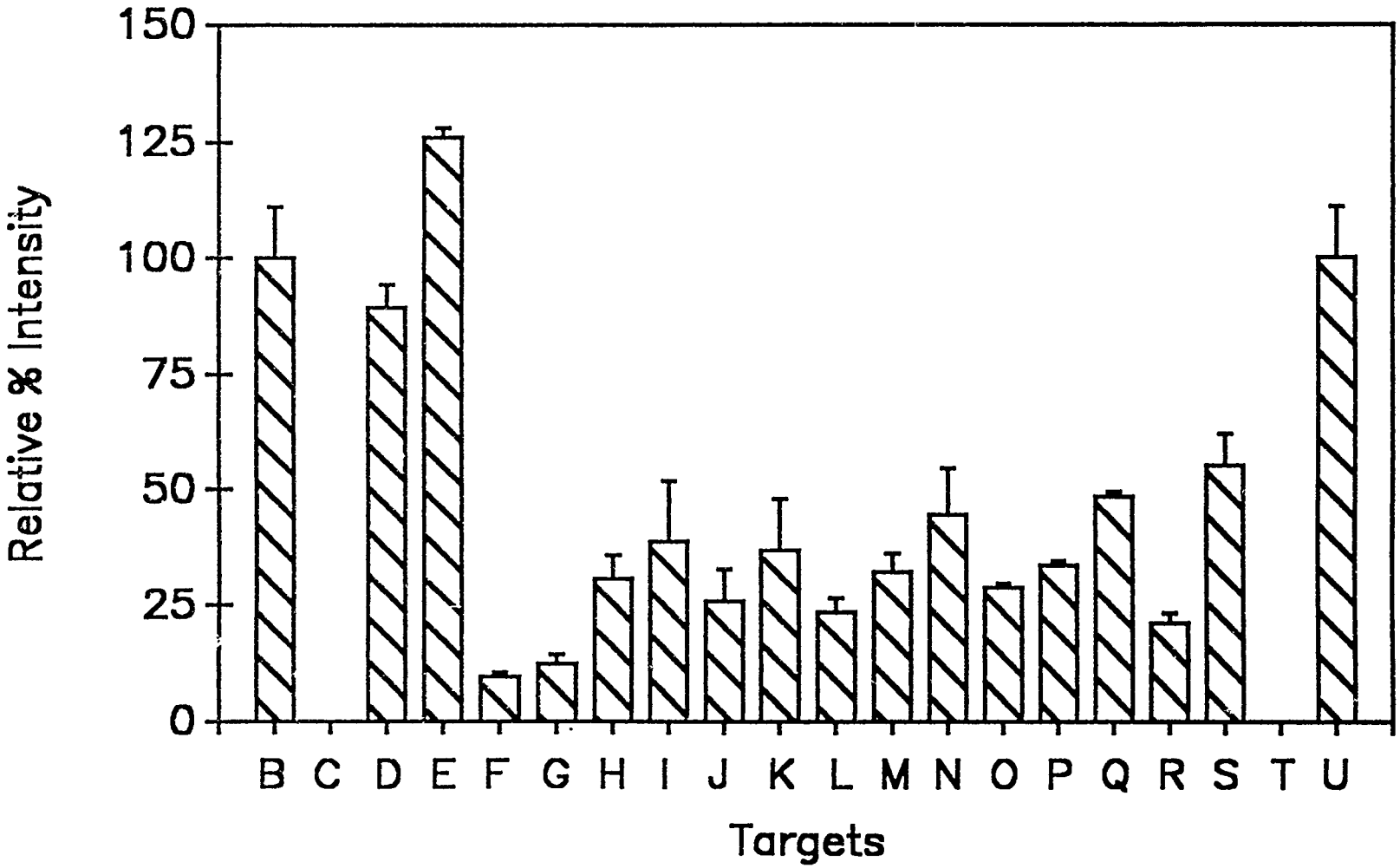



Fig-2


150
125
100
75
50
25
0
Relative % Intensity
B C D E F G H I J K L M N O P Q R S T U
Targets

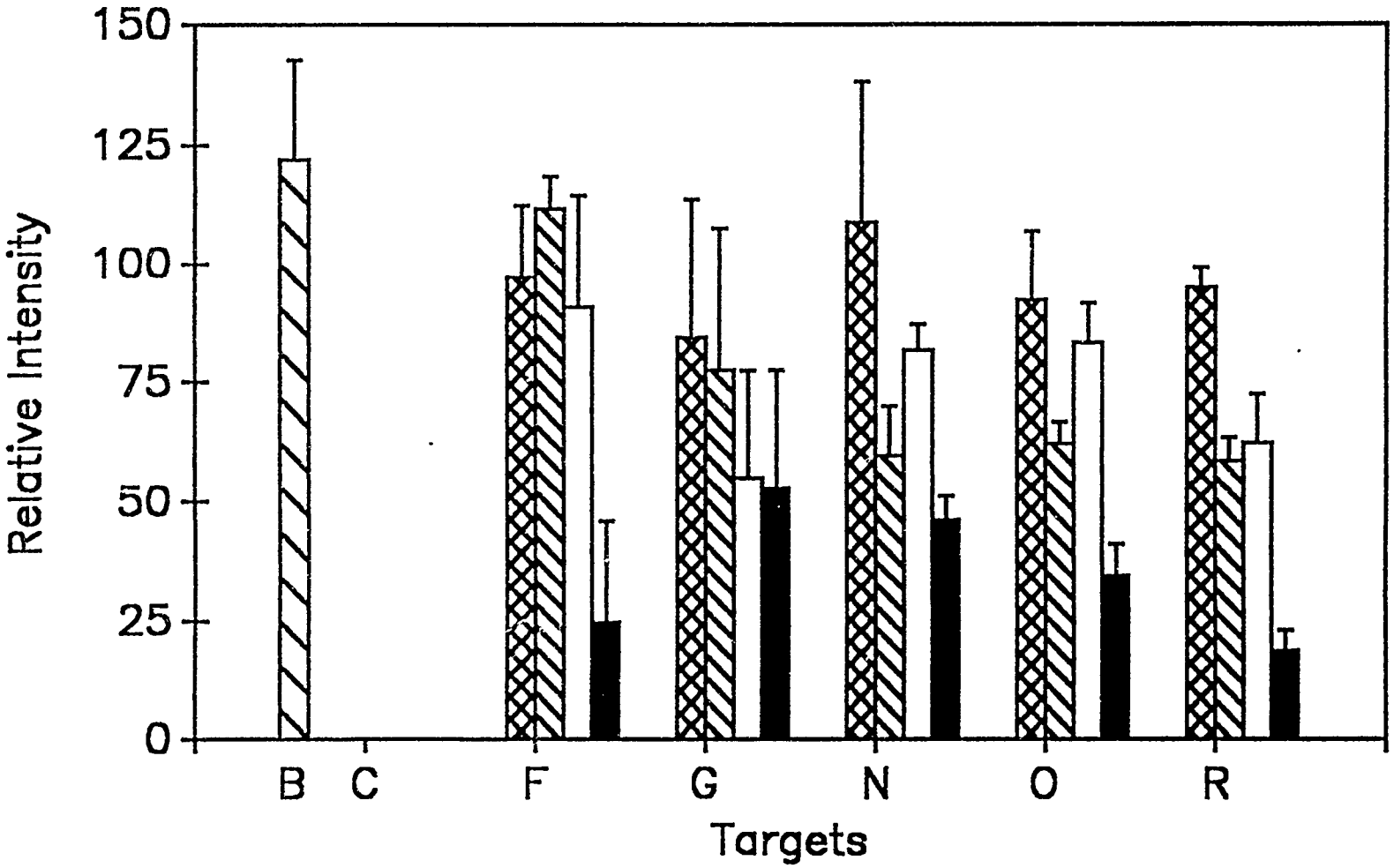



Fig. 4

Peptide Research

# Role of the Proline Residues on the Immunogenic Properties of a *P. falciparum* Circumsporozoite Peptide Linked to a Carrier Protein

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ABSTRACT

*The circumsporozoite (CS) protein of P. falciparum contains an immunodominant epitope, NANP, that is repeated 37 times in the native molecule. The presence of proline in the coat proteins of the Plasmodium parasite at various developmental stages and strains is a frequent occurrence. In this study we evaluate the influence of substitution of proline residues by glycine on the immunogenic behavior of two tandemly repeated peptides linked via glutaraldehyde to a protein carrier. The (NANP)4 P. falciparum circumsporozoite peptide and its glycine-substitute analog, (NANG)4. The results obtained show that the (NANP)4 induces antibodies which recognize the peptide free in solution, bound on a solid phase, and linked to a carrier protein. It has been previously reported that such antibodies recognize the antigenic sites of the peptide in the native protein on the surface of the sporozoite. Antibodies raised against (NANG)4 in the same experimental conditions as (NANP)4, cannot recognize the peptide free in solution or bound to the solid phase. However, these antibodies can react with the peptide when it is linked to a protein carrier. The coupling of a glycine-containing analog to a carrier results in a significant shift in its conformation, allowing it to be recognized by the antibodies.*

## INTRODUCTION

Studies to date have indicated that antibodies generated against peptides containing 12 to 40 amino acid residues of the repetitive region of the circumsporozoite (CS) protein are capable of reacting with sporozoite and neutralizing its infectivity *in vitro* (1,13,14). However, small peptides are weakly immunogenic because of their low molecular size. An increase in their immunogenicity is obtained by conjugating them to protein carriers. Conjugation is carried out by means of bifunctional reagents that can form a covalent link between the peptide and the carrier molecule. Two types of reagents are most frequently used. Glutaraldehyde (g) and carbodiimide (c). Attachment of the peptide to a carrier protein that provides a different environment than the peptide alone may influence its conformational behavior (5) and consequently its presentation to the immune system. In this study we attempt to evaluate the influence of substitution of proline residues by glycine on the immunogenic behavior of two tandemly repeated peptides linked via glutaraldehyde to tetanus toxoid (TT) carrier: The $(NANP)_4$ *P. falciparum* circumsporozoite peptide and its glycine-substituted analog, $(NANG)_4$. To this end we synthesized the native tandem repeat repeated 4 and 8 times, i.e., $(NANP)_{4,8}$, as well as its glycine-analog $(NANG)_{4,8}$, in which the proline residues were replaced by glycine. The $(NANP)_4$ and $(NANG)_4$ peptides were each conjugated via glutaraldehyde to TT and administered in four strains of mice. Our results indicate that the immunogenic behavior of the two peptide haptens differs. Antibodies raised against $(NANP)_4$ recognize $(NANP)_8$ free, bound on a solid phase and linked to a protein carrier. In a previous report, we showed that such antibodies recognize the sporozoite and neutralize its infectivity *in vitro*. In contrast, antibodies raised against the $(NANG)_4$ cannot recognize $(NANG)_8$ peptide, free or bound on a solid phase. However, anti $(NANG)_4$ antibodies can bind to $(NANG)_8$ when it is linked to an unrelated bovine serum albumin carrier (BSA).

## MATERIALS AND METHODS

### Peptide Synthesis

Derivatized amino acid (t-Boc, tert-butyloxycarboxyl) were of the L configuration and were purchased from Bachem (Torrance, CA). Boc-Asn was in the form of its nitrophenylester. Syntheses were carried out using a benzhydrylamine resin (0.654 meq/g, Beckman Instruments (Palo Alto, CA) on a Vega (Tuscon, AZ) model 250C synthesizer controlled by an Apple IIe computer with a program based on a modification of the Merrifield method (16). A 2-g sample of the benzhydrylamine resin was suspended and washed 3 times with methylene chloride, 3 times with ethanol, and 3 times with $CH_2Cl_2$ in the synthesizer. The resin was washed 2 min with 50% trifluoroacetic acid (TFA) (containing 10% anisole in $CH_2Cl_2$) and then treated with 50% trifluoroacetic acid (containing 10% anisole in $CH_2Cl_2$) for 30 min, washed 1½ times with $CH_2Cl_2$, and neutralized by washing twice with 10% diisopropylethylamine in $CH_2Cl_2$. The first Boc-amino acid was coupled for 1 h to the benzhydrylamine resin by using a 3 molar excess of dicyclocarbodiimide and hydroxybenzotriazole in $CH_2Cl_2$ and a 3-fold molar excess of

diisopropylethylamine. Then, another aliquot of hydroxybenzotriazole and diisopropylethylamine was added at a 2-fold molar excess for an additional 1 h. Following coupling, the resin was washed with $CH_2Cl_2$ (three washes), absolute ethanol (three washes), and $CH_2Cl_2$ (three washes), and an aliquot of the mixture was then tested by using the Kaiser ninhydrin procedure (10) to test for completion of coupling of the Boc-amino acid to the growing peptide chain.

### Cleavage and Extraction of Peptide from the Benzhydrylamine Resin

Cleavage of each of the peptide resins (2 g of each) was performed in a Peninsula (San Carlos, CA) HF apparatus in the presence of anisole (1.2 ml/mg of peptide-resin) and methyl ethyl sulfide (1 ml/mg) at 0° C for 1 h, after which the mixture was thoroughly dried under high vacuum. The mixture was then washed with cold anhydrous ether, extracted with alternate washes of water and glacial acetic acid, and then lyophilized.

### Purification of Crude Synthetic Product

Crude synthetic peptides were desalted and purified by gel filtration on a column of Sephadex G-25 (120 x 2.0 cm) equilibrated with 0.1 M $NH_4HCO_3$, pH 8.0. Column effluent was monitored by the UV absorbance at 254 and 206 nm with an LKB (Piscataway, NJ) UV-Cord III monitor.

### Preparation of Conjugates

**Glutaraldehyde conjugates, (g).** The $NH_2$ groups of the protein and the peptide react with the aldehyde group of glutaraldehyde to make a Schiff base. Since glutaraldehyde is a bifunctional reagent, there is formation of inter- and intramolecular cross-linkages.

**Coupling of peptide to carrier protein.** The following conjugates were prepared using glutaraldehyde to couple peptides via their $NH_2$ groups to the carrier protein: TT(NANP)$_4$(g), TT(NANG)$_4$ (g), BSA(NANP)$_4$(g), and BSA (NANG)$_8$(g). The amounts of peptide and carrier were calculated to give approximately 1.2 peptide $NH_2$ equivalents for each amino group on the carrier. The coupling reaction was carried out at 20° C in 0.1 M sodium bicarbonate, pH 8, with glutaraldehyde at 2.63 mM. The reaction was allowed to proceed for 6 days with constant stirring, followed by dialysis against phosphate-buffered saline (PBS).

**Preparation of carrier protein-free conjugates.** Polymerization of (NANG)$_8$ [poly(NANG)$_8$(g)], copolymerization of (NANG)$_8$ with an excess of lysine [poly(NANG)$_8$lys(g)] and copolymerization of (NANG)$_8$ with an excess of glycine [poly (NANG)$_8$gly(g)] was achieved by treatment with glutaraldehyde under similar conditions.

**Carbodiimide conjugates, (c).** BSA(NANG)$_8$(c) was prepared as follows: (NANG)$_8$ peptide (1.0 mM) and BSA (0.03 mM) were mixed in 0.1 M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide HCl for 10 h at 22° C, pH 5.0. An equal volume of 1.0 M glycine was then added. The mixture was rotated overnight at 4° C and then dialyzed extensively against PBS.

The conjugates were analyzed using high performance size exclusion chromatography [TSK G3000 SW column (LKB), isocratic elution with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl and amino acid analysis.

### Amino Acid Analysis

Samples were hydrolyzed in 5.7 N HCl for 22 h at 100° C, dried, reconstituted, and applied to the amino acid analyzer (Applied Biosystems, Foster City, CA, model 420 A derivatizer). Amino acids were quantified as their phenylthiocarbamyl derivatives on an Applied Biosystems Model 130A HPLC.

### Determination of Peptide/Carrier Ratio of Conjugates

This ratio was determined by comparing the amino acid analysis of the conjugate to the carrier alone, according to a method of calculation described by Briand et al. (3).

### Immunization by Peptide-Carrier Conjugates

Female Swiss, DBA/2, BALB/C, and C57B1/6 mice, 7 weeks of age, were purchased from Harlan Company (Madison, WI). Mice (eight per group) were injected subcutaneously with 50 µg of protein or peptide-carrier conjugate in the presence of aluminum hydroxide (Al(OH)$_3$) (100 µg per mouse). Mice were boosted 30 days later with 50 µg of peptide-carrier conjugate in absence of adjuvant. Sera were collected by retroorbital bleeding at weekly intervals after the first injection and stored at -20° C before titration.

### Antibody Titration

Antibody titers against the synthetic peptides or the carriers were measured by enzyme linked immunosorbent assay (ELISA) according to experimental conditions described previously (9). Titer plate wells (Nunc immunoplate, Denmark) were coated with 10 µg peptide or 4 µg protein per ml. After incubation for 2 h at 37° C, the plates were washed and incubated for 1 h with serial dilutions of sera at the same temperature. The wells were then washed and treated with a rabbit anti-mouse immunoglobulin G (IgG)-peroxidase conjugate (Miles Laboratories, Naperville, IL) for an additional hour at 37° C. Twelve min after the addition of the substrate-containing solution, the reaction was stopped with 50 µl of 12% $H_2SO_4$. Optical density was determined with a spectrophotometer reader (Titertek Multiskan Plus, Mclean, VA). ELISA titers were expressed as the maximum dilution giving a two-fold higher absorbance than the control serum diluted at 1:100. Control sera were obtained from mice which received only Al(OH)$_3$. Preliminary experiments using a monoclonal antibody (5508) and a series of peptides from (NANP)$_2$ to (NANP)$_8$ demonstrated that (NANP)$_8$ bound to the antibodies better than the (NANP)$_4$ peptide (unpublished). Consequently, we chose (NANG)$_8$ as the coating antigen to measure the anti (NANG)$_4$ antibodies.

### ELISA Inhibition Tests

Inhibition studies were performed on sera diluted in 1% BSA, 0.1% Tween 20, at a level of 1 ± 0.3 O.D. when tested against the antigen bound in the plate. The inhibiting antigens were incubated with the immune sera at the concentrations indicated under Results. After 20 h at 4° C the sera were tested by ELISA according to the same conditions.

### Circular Dichroism (CD) Studies

CD measurements were performed on a Jasco 500A spectropolarimeter (Japan), using a cylindrical quartz cell of 0.1-cm path length. The instrument

was normally used at a sensitivity of 30 μA units/cm. Peptides were dissolved in phosphate buffer saline, pH 7, at a concentration of 110 μg/ml. Mean residue ellipticities [Θ] were calculated from the expression:

$$[\Theta] = \frac{A.S.MRW.3300}{C.L.10} \quad (deg.cm^2.dmol^{-1})$$

where A is the observed dichroic absorbance at wavelength λ (cm), S is the sensitivity setting (A units $cm^{-1}$), MRW is the mean residue molecular weight, C is the peptide concentration in g/dl and L is the optical path length in cm. All measurements were made at 20° C.

## RESULTS

### Antibody Measurement with $(NANP)_8$ and $(NANG)_8$ Peptides

The antibody response of four strains of mice immunized with $TT(NANP)_4(g)$ and $TT(NANG)_4(g)$ was analyzed by ELISA. As shown in Figure 1, a response to an antibody raised against $(NANP)_4$ was detected using $(NANP)_8$ as the solid phase antigen. In contrast, no antibodies against the $(NANG)_4$ peptide could be detected when $(NANG)_8$ peptide was used as the solid phase antigen. However, as shown in Table 1, when the mice were immunized with $BSA(NANG)_8(c)$, an antibody response against $(NANG)_4$ was detected using $(NANG)_8$. The antibody titer to $(NANG)_8$ was comparable to the antibody titer obtained with $TT(NANG)_4$, indicating that $(NANG)_8$ can be used as antigen in the solid phase assay. However, $BSA(NANG)_8$ (c) induced a lower antibody response than $TT(NANG)_4(g)$.

### Antibody Measurement with $(NANG)_8$ Linked to BSA Carrier

The $(NANG)_8$ peptide was linked to BSA by either glutaraldehyde [$BSA(NANG)_8(g)$] or by carbodiimide [$BSA(NANG)_8(c)$] to improve its presentation to the antibodies in the solid phase assay. The results in Figure 2 show a stronger binding of the antibodies to $BSA(NANG)_8(g)$ than to $BSA(NANG)_8(c)$. The antibodies raised against $TT(NANG)_4(g)$ recognized $(NANG)_8$ linked to BSA but not directly bound in the plate. These results suggest two hypotheses: 1) The antibodies recognize conformations in the peptide linked to a protein carrier which are not present in the peptide when free in

Table 1. Secondary Antibody Response of Mice Immunized with the [$BSA(NANG)_8(c)$] Conjugate

| Treatment Day 0 | ELISA Titer of Antibody Against: $(NANG)_8$ | $TT(NANG)_4(g)$ | BSA | TT |
|---|---|---|---|---|
| [$BSA(NANG)_8(c)$] with $Al(OH)_3$ | 1,200 | 1,150 | 14,800 | <100 |

Eight Swiss mice received 50 μg of $BSA(NANG)_8(c)$ conjugate with 100 μg of $Al(OH)_3$ on day 0. The mice were boosted on day 30 with the same amount of conjugate in saline solution.

Table 2. Inhibitory Activity of Peptides $(NANG)_4$ and $(NANG)_8$ on the Binding of Anti $TT(NANG)_4(g)$ Antibodies to $BSA(NANG)_8(g)$ Conjugate

| Inhibitors | Peptide/Carrier Molar Ratio | Molar Concentration of Peptides Necessary for 50% Inhibition of Antibody Binding to $BSA(NANG)_8(g)$ Conjugate |
|---|---|---|
| $(NANG)_8$ | - | - |
| $BSA(NANG)_8(g)$ | 16 | $1.7\ 10^{-9}$ |
| $TT(NANG)_4(g)$ | 23 | $2\ 10^{-9}$ |
| $Poly(NANG)_8(g)$ | - | $4\ 10^{-9}$ |
| $Poly(NANG)_8lys(g)$ | - | $4\ 10^{-9}$ |

Molar concentration of the peptide in the peptide-TT conjugate was calculated from quantitative analysis after total acid hydrolysis of an aliquot of the solution of conjugate.

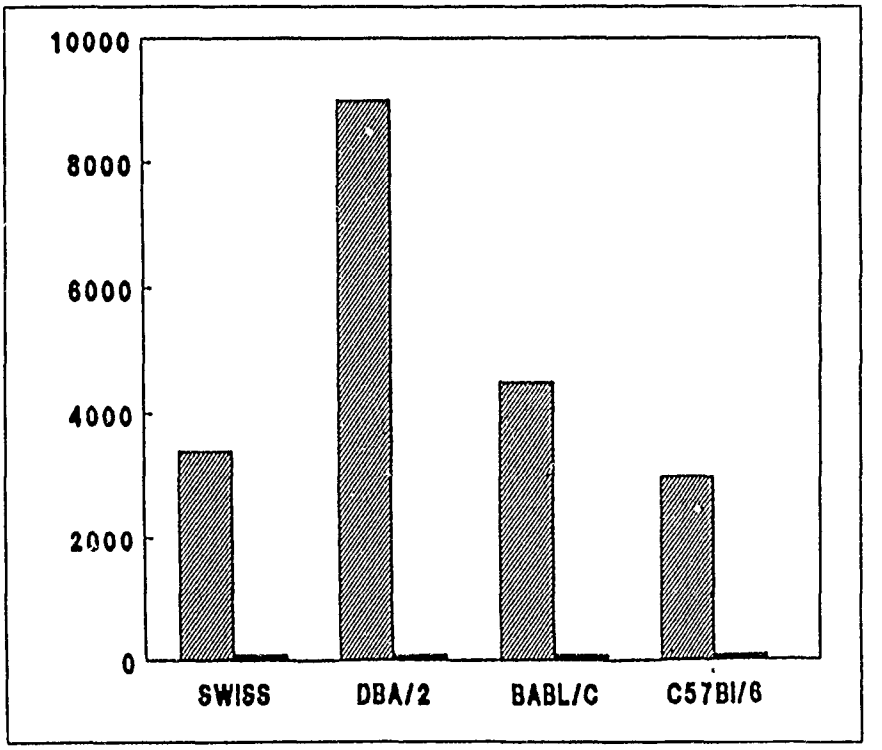


Figure 1. Antipeptide antibody response of four strains of mice immunized with $TT(NANP)_4(g)$ (cross-hatched bars) and $TT(NANG)_4(g)$ (solid bars). Antibody titers were measured on $(NANP)_8$ and $(NANG)_8$, respectively.

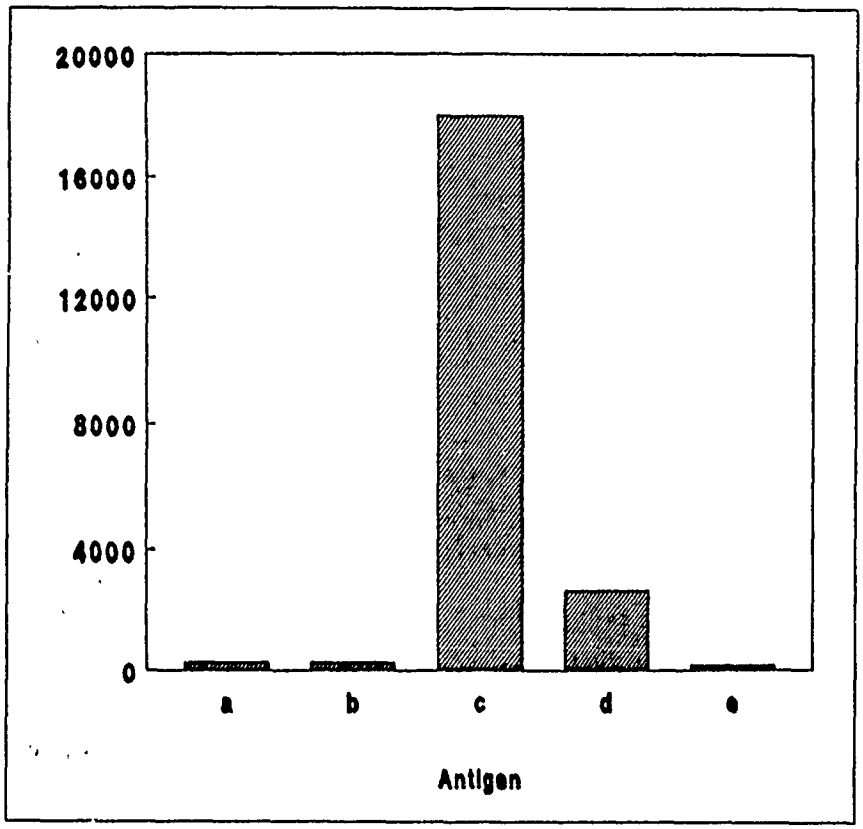


**Figure 2. Antibody response of Swiss mice immunized with TT(NANG)4(g), measured with different antigens.** a) $(NANG)_4$, b) $(NANG)_8$, c) $BSA(NANG)_8(g)$, d) $BSA(NANG)_8(c)$, and e) BSA.

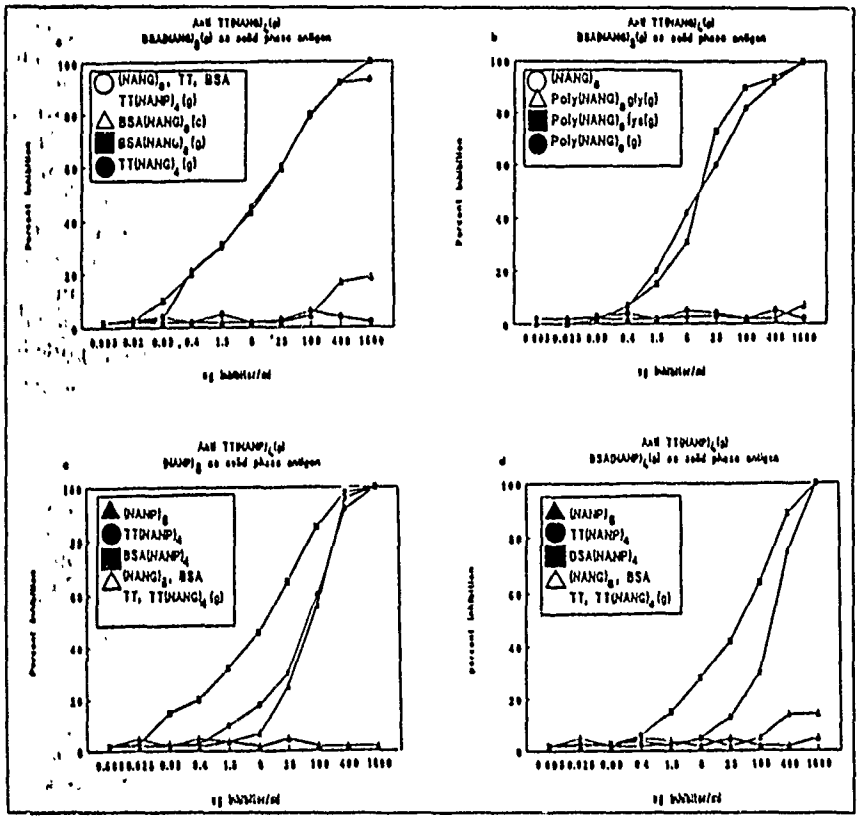

**Figure 3. ELISA inhibition experiments.** Inhibiting antigens were incubated 18 h at 4° C, at the concentrations indicated, with sera diluted at the level 1 ± 0.2 O.D. when tested against the solid phase antigen. Graphs a-d represent inhibition studies performed on pooled immune sera obtained with the immunogen indicated.

solution or bound on the solid phase; 2) the antibodies recognize the glutaraldehyde bound between the lysine of the carrier protein and the N terminal amino part of the peptide. In order to evaluate each of these hypotheses ELISA inhibition experiments were performed.

## Specificity of the Antibody Binding to BSA(NANG)8(g)

The specificity of antibody toward the plate-bound BSA(NANG)8(g) antigen was examined by ELISA competitive inhibition analysis. As shown in Figure 3a, the binding of antibody to antigen was inhibited by BSA (NANG)8(g) and TT(NANG)4(g). Only a partial inhibition (18%) of the antibody binding was achieved with BSA(NANG)8(c). Conversely, neither $(NANG)_8$, BSA, TT, nor TT(NANP)4 (g) inhibited the binding. Three carrier-free glutaraldehyde compounds containing $(NANG)_8$ were also studied for their capacity to inhibit antibody binding to BSA(NANG)8(g). For this purpose, conjugates were prepared using poly(NANG)8lys(g), poly(NANG)8gly (g) and poly(NANG)8(g). Complete inhibition was achieved with the poly(NANG)8(g) and poly(NANG)8lys (g) conjugates, but there was no inhibition using poly(NANG)8gly(9) (Figure 2b). The degree of inhibition of binding by $(NANG)_4$ or $(NANG)_8$ in the different $(NANG)_8$ conjugates is shown in Table 2. The carrier-free $(NANG)_8$ conjugates were two times more efficient than the peptide-carrier conjugates. The fact that poly $(NANG)_8(g)$ and poly(NANG)8lys(g) can both inhibit the antibody binding to BSA(NANG)8(g) with the same efficiency shows that lysine is not important in the antibody sites.

## Specificity of the Antibody Binding to (NANP)8 and TT(NANP)4(g)

The specificity of the antipeptide antibodies raised against TT (NANP)4(g) was examined by ELISA competitive inhibition analysis using either $(NANP)_8$ or BSA(NANP)4(g) as solid phase antigens. As shown in Figure 3c, a complete inhibition of antibody binding to $(NANP)_8$ was achieved with $(NANP)_8$, BSA (NANP)4(g), and TT(NANP)4(g). Neither BSA, TT, nor TT(NANG)4(g) inhibited the binding. Conversely, when BSA(NANP)4(g) (Figure 3d) was used as solid-phase antigen, only a

partial inhibition (14%) was achieved with $(NANP)_8$, whereas a complete inhibition was obtained with BSA $(NANP)_4(g)$ and TT$(NANP)_4(g)$. These results suggest that most of the antibodies which recognized the peptide linked to the carrier protein could not bind to the peptide alone.

### Circular Dichroism on $(NANP)_8$, $(NANG)_{4,8}$, and $(NANG)_8$ Derivatives

The CD spectra between 200 and 250 nm in phosphate-buffered saline solution for $(NANG)_4$ and $(NANG)_8$ are shown on Figure 4a. These two peptides have spectra typical of largely randomly coiled structures with a minimum at about 202 nm and a less resolved shoulder from 210 to 230 nm (2,8). The spectrum of $(NANP)_8$ (Figure 4b) also exhibits the same minimum band at 202 nm, but not the shoulder around 220 nm. The poly $(NANG)_8$gly(g) conjugate (Figure 4a), which exhibits a sepctrum similar to those of $(NANG)_4$ and $(NANG)_8$, also shows the same immunogenic behavior as $(NANG)_4$ and $(NANG)_8$, since it cannot inhibit the binding of antibodies raised against TT$(NANG)_4(g)$ to BSA $(NANG)_8(g)$. Conversely, the spectra of poly$(NANG)_8(g)$ and poly $(NANG)_8$lys(g) in Figure 4b are similar to the $(NANP)_8$ spectrum in that they lack the shoulder around 220 nm. These two $(NANG)_8$ conjugates are both able to inhibit the antibody binding to BSA$(NANG)_8(g)$.

The spectra of these different compounds can be categorized in two separate groups. Group I is characterized by a shoulder at 220 nm, group II by the absence of these features. The presence or absence of the shoulder represents a small but significant difference between these two groups. The disappearance of the shoulder at 220 nm could be ascribed to a complete lack of ordered secondary structure in the poly$(NANG)_8(g)$ and poly $(NANG)_8$lys(g) conjugates, allowing the peptide to react effectively with the antibodies raised against TT $(NANG)_4(g)$.

## DISCUSSION

Antipeptide antibodies generated by TT$(NANP)_4(g)$ immunization bind to $(NANP)_8$ when $(NANP)_8$ is used as coating antigen in the solid phase assay. It has been shown previously that these antipeptide antibodies can recognize the sporozoite in an immunofluorescence antibody test and inhibit the sporozoite penetration into cultured hepatocytes (13,14). We show in the present study that antibodies raised against TT$(NANP)_4(g)$ recognize the peptide in solution, bound in the plate, and glutaraldehyde-treated TT (data not shown) do not inhibit the antibody binding to BSA $(NANG)_8(g)$. Furthermore, poly $(NANG)_8$ which lacks lysine is as efficient an inhibitor as poly $(NANG)_8$ lys(g). On the other hand, poly $(NANG)_8$gly(g) cannot inhibit the antibody binding.

The presence of proline residues may tend to fix the polypeptide backbone (18). Replacement of proline by glycine may increase the rotational freedom of the peptide. However, attachment of the peptide to a carrier protein which provides a different environment than the peptide alone may allow only some conformational states. The CD data show that the peptide in poly$(NANG)_8(g)$ and poly$(NANG)_8$ lys(g) may present the same conformation as the peptide in the peptide-carrier conjugates.

It has been demonstrated previously that peptides with proline in the i+1 and Asn in the i+2 position readily form protein β turns (12). The *P. falciparum* sporozoite immunodominant epitope contains repeating pro-asn sequences and the *P. knowlesi* sporozoite immunodominant epitope contains progln sequences, suggesting that these repeats may possess a cross-beta backbone (7). Another piece of evidence for this conformation is the discrepancy between the molecular weight of *P. knowlesi* sporozoite CS protein obtained by DNA sequencing (36,700 daltons) and that obtained by estimate on SDS-polyacrylamide gel electrophoresis (52,000 daltons). The presence of proline in the tandem repeats in a number of stages and species of the plasmodium parasite is rather common. For example, proline appears in the S-antigen of *P. falciparum* (4), the FIRA merozoite repeat (19), the histidine-rich knob proteins (11), the *P. vivax* CS protein (15), the *P. knowlesi* CS protein (nuri strain) (17), and the *P. berghei* CS protein (6). The $(NANP)_{4,8}$ peptides in solution, bound in the ELISA plate, or linked via glutaraldehyde to a carrier protein adopt, with a significant frequency, a conformation compatible with that of the cognate site in the circumsporozoite protein. Thus, the presence of proline in repetitive sequences may represent an efficient tool to define peptides eliciting antibodies which recognize directly the peptide in

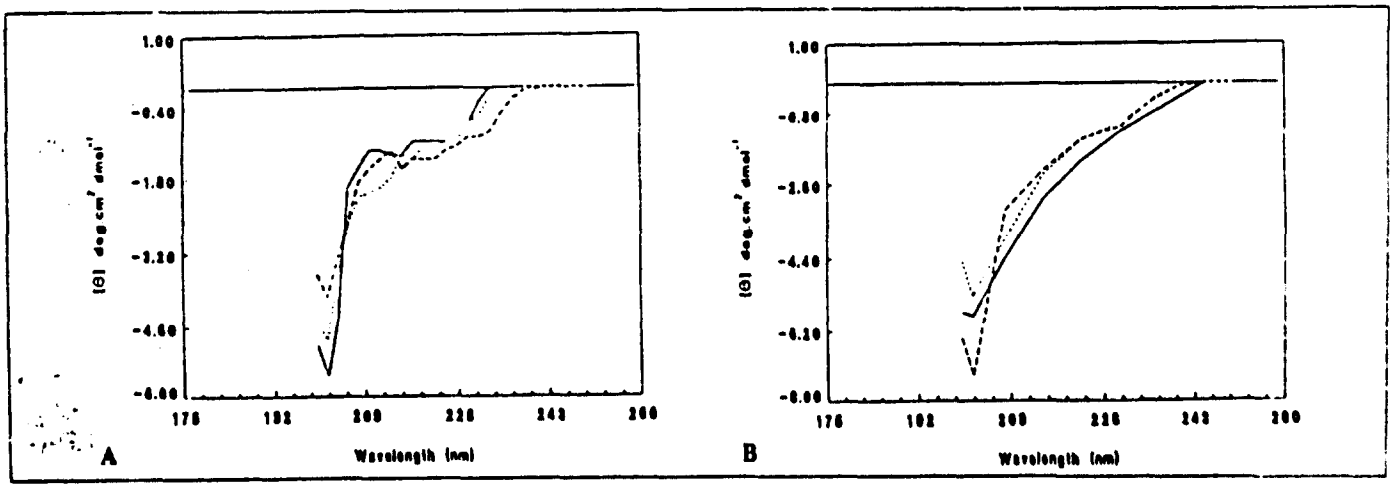


**Figure 4a.** CD spectra of: $(NANG)_4$ ——, $(NANG)_8$ ------, Poly$(NANG)_8$gly(g) – – – – in PBS pH 7, 22° C. **Figure 4b.** CD spectra of: $(NANP)_8$ ——, Poly$(NANG)_8(g)$ ------, Poly$(NANG)_8$lys(g) – – – – in PBS pH 7, 22° C.

the solid phase assay and reacting with the native protein at the surface of the pathogen.

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